

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
22 April 2004 (22.04.2004)

PCT

(10) International Publication Number  
**WO 2004/033651 A2**

- (51) International Patent Classification<sup>7</sup>: C12N  
Grove, PA 19090 (US). CHEN, Xi [CN/US]; 107 Whitney Place, Lansdale, PA 19446 (US).
- (21) International Application Number: PCT/US2003/031974 (74) Agents: DOYLE, Kathryn et al.; Morgan, Lewis & Bockius L.L.P., 1701 Market Street, Philadelphia, PA 19103 (US).
- (22) International Filing Date: 8 October 2003 (08.10.2003)
- (25) Filing Language: English (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (26) Publication Language: English (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (30) Priority Data:  
PCT/US02/32263 9 October 2002 (09.10.2002) US  
10/287,994 5 November 2002 (05.11.2002) US  
10/360,770 6 January 2003 (06.01.2003) US  
10/360,779 19 February 2003 (19.02.2003) US  
10/410,945 9 April 2003 (09.04.2003) US
- (71) Applicant (*for all designated States except US*): NEOSE TECHNOLOGIES, INC. [US/US]; 102 Witmer Road, Horsham, PA 19044 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): DE FREES, Shawn [US/US]; 126 Filly Drive, North Wales, PA 19454 (US). ZOPE, David [US/US]; 560 W. Beechtree Lane, Wayne, PA 19087 (US). BAYER, Robert [US/US]; 6105 Dirac Street, San Diego, CA 92122 (US). BOWE, Caryn [US/US]; 310 Maple Avenue, Doylestown, PA 18901 (US). HAKES, David [US/US]; 14 Fern Avenue, Willow
- Published:**  
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 2004/033651 A2

(54) Title: ERYTHROPOIETIN: REMODELING AND GLYCOCONJUGATION OF ERYTHROPOIETIN

(57) Abstract: The invention includes methods and compositions for remodeling a peptide molecule, including the addition or deletion of one or more glycosyl groups to a peptide, and/or the addition of a modifying group to a peptide.

## TITLE OF THE INVENTION

ERYTHROPOIETIN: REMODELING AND GLYCOCONJUGATION OF  
ERYTHROPOIETIN

## 5 BACKGROUND OF THE INVENTION

Most naturally occurring peptides contain carbohydrate moieties attached to the peptide via specific linkages to a select number of amino acids along the length of the primary peptide chain. Thus, many naturally occurring peptides are termed "glycopeptides." The variability of the glycosylation pattern on any given peptide has enormous implications  
10 for the function of that peptide. For example, the structure of the N-linked glycans on a peptide can impact various characteristics of the peptide, including the protease susceptibility, intracellular trafficking, secretion, tissue targeting, biological half-life and antigenicity of the peptide in a cell or organism. The alteration of one or more of these characteristics greatly affects the efficacy of a peptide in its natural setting, and also affects  
15 the efficacy of the peptide as a therapeutic agent in situations where the peptide has been generated for that purpose.

The carbohydrate structure attached to the peptide chain is known as a "glycan" molecule. The specific glycan structure present on a peptide affects the solubility and aggregation characteristics of the peptide, the folding of the primary peptide chain and  
20 therefore its functional or enzymatic activity, the resistance of the peptide to proteolytic attack and the control of proteolysis leading to the conversion of inactive forms of the peptide to active forms. Importantly, terminal sialic acid residues present on the glycan molecule affect the length of the half life of the peptide in the mammalian circulatory system. Peptides whose glycans do not contain terminal sialic acid residues are rapidly removed from the  
25 circulation by the liver, an event which negates any potential therapeutic benefit of the peptide.

The glycan structures found in naturally occurring glycopeptides are typically divided into two classes, N-linked and O-linked glycans.

Peptides expressed in eukaryotic cells are typically N-glycosylated on asparagine  
30 residues at sites in the peptide primary structure containing the sequence asparagine-X-



serine/threonine where X can be any amino acid except proline and aspartic acid. The carbohydrate portion of such peptides is known as an N-linked glycan. The early events of N-glycosylation occur in the endoplasmic reticulum (ER) and are identical in mammals, plants, insects and other higher eukaryotes. First, an oligosaccharide chain comprising  
5 fourteen sugar residues is constructed on a lipid carrier molecule. As the nascent peptide is translated and translocated into the ER, the entire oligosaccharide chain is transferred to the amide group of the asparagine residue in a reaction catalyzed by a membrane bound glycosyltransferase enzyme. The N-linked glycan is further processed both in the ER and in the Golgi apparatus. The further processing generally entails removal of some of the sugar  
10 residues and addition of other sugar residues in reactions catalyzed by glycosidases and glycosyltransferases specific for the sugar residues removed and added.

Typically, the final structures of the N-linked glycans are dependent upon the organism in which the peptide is produced. For example, in general, peptides produced in bacteria are completely unglycosylated. Peptides expressed in insect cells contain high  
15 mannose and paucimannose N-linked oligosaccharide chains, among others. Peptides produced in mammalian cell culture are usually glycosylated differently depending, e.g., upon the species and cell culture conditions. Even in the same species and under the same conditions, a certain amount of heterogeneity in the glycosyl chains is sometimes encountered. Further, peptides produced in plant cells comprise glycan structures that differ  
20 significantly from those produced in animal cells. The dilemma in the art of the production of recombinant peptides, particularly when the peptides are to be used as therapeutic agents, is to be able to generate peptides that are correctly glycosylated, i.e., to be able to generate a peptide having a glycan structure that resembles, or is identical to that present on the naturally occurring form of the peptide. Most peptides produced by recombinant means  
25 comprise glycan structures that are different from the naturally occurring glycans.

A variety of methods have been proposed in the art to customize the glycosylation pattern of a peptide including those described in WO 99/22764, WO 98/58964, WO 99/54342 and U.S. Patent No. 5,047,335, among others. Essentially, many of the enzymes required for the *in vitro* glycosylation of peptides have been cloned and sequenced. In some instances,  
30 these enzymes have been used *in vitro* to add specific sugars to an incomplete glycan molecule on a peptide. In other instances, cells have been genetically engineered to express a

combination of enzymes and desired peptides such that addition of a desired sugar moiety to an expressed peptide occurs within the cell.

Peptides may also be modified by addition of O-linked glycans, also called mucin-type glycans because of their prevalence on mucinous glycopeptide. Unlike N-glycans that are linked to asparagine residues and are formed by *en bloc* transfer of oligosaccharide from lipid-bound intermediates, O-glycans are linked primarily to serine and threonine residues and are formed by the stepwise addition of sugars from nucleotide sugars (Tanner *et al.*, *Biochim. Biophys. Acta.* 906:81-91 (1987); and Hounsell *et al.*, *Glycoconj. J.* 13:19-26 (1996)). Peptide function can be affected by the structure of the O-linked glycans present thereon. For example, the activity of P-selectin ligand is affected by the O-linked glycan structure present thereon. For a review of O-linked glycan structures, see Schachter and Brockhausen, *The Biosynthesis of Branched O-Linked Glycans*, 1989, Society for Experimental Biology, pp. 1-26 (Great Britain). Other glycosylation patterns are formed by linking glycosylphosphatidylinositol to the carboxyl-terminal carboxyl group of the protein (Takeda *et al.*, *Trends Biochem. Sci.* 20:367-371 (1995); and Udenfriend *et al.*, *Ann. Rev. Biochem.* 64:593-591 (1995).

Although various techniques currently exist to modify the N-linked glycans of peptides, there exists in the art the need for a generally applicable method of producing peptides having a desired, i.e., a customized glycosylation pattern. There is a particular need in the art for the customized *in vitro* glycosylation of peptides, where the resulting peptide can be produced at industrial scale. This and other needs are met by the present invention.

The administration of glycosylated and non-glycosylated peptides for engendering a particular physiological response is well known in the medicinal arts. Among the best known peptides utilized for this purpose is insulin, which is used to treat diabetes. Enzymes have also been used for their therapeutic benefits. A major factor, which has limited the use of therapeutic peptides is the immunogenic nature of most peptides. In a patient, an immunogenic response to an administered peptide can neutralize the peptide and/or lead to the development of an allergic response in the patient. Other deficiencies of therapeutic peptides include suboptimal potency and rapid clearance rates. The problems inherent in peptide therapeutics are recognized in the art, and various methods of eliminating the

problems have been investigated. To provide soluble peptide therapeutics, synthetic polymers have been attached to the peptide backbone.

Poly(ethylene glycol) ("PEG") is an exemplary polymer that has been conjugated to peptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides and prolong the clearance time from the circulation. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) concerns non-immunogenic peptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole peptide and at least 15% of the physiological activity is maintained.

WO 93/15189 (Veronese *et al.*) concerns a method to maintain the activity of polyethylene glycol-modified proteolytic enzymes by linking the proteolytic enzyme to a macromolecularized inhibitor. The conjugates are intended for medical applications.

The principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. For example, U.S. Patent No.

4,088,538 discloses an enzymatically active polymer-enzyme conjugate of an enzyme covalently linked to PEG. Similarly, U.S. Patent No. 4,496,689 discloses a covalently attached complex of  $\alpha$ -1 protease inhibitor with a polymer such as PEG or methoxypoly(ethylene glycol) ("mPEG"). Abuchowski *et al.* (*J. Biol. Chem.* **252**: 3578 (1977) discloses the covalent attachment of mPEG to an amine group of bovine serum

albumin. U.S. Patent No. 4,414,147 discloses a method of rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride). PCT WO 87/00056 discloses conjugation of PEG and poly(oxyethylated) polyols to such proteins as interferon- $\beta$ , interleukin-2 and immunotoxins. EP 154,316 discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of the lymphokine. U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as a polysaccharide.

Another mode of attaching PEG to peptides is through the non-specific oxidation of glycosyl residues on a peptide. The oxidized sugar is utilized as a locus for attaching a PEG moiety to the peptide. For example, M'Timkulu (WO 94/05332) discloses the use of a hydrazine- or amino-PEG to add PEG to a glycoprotein. The glycosyl moieties are randomly

oxidized to the corresponding aldehydes, which are subsequently coupled to the amino-PEG. See also, Bona et al. (WO 96/40731), where a PEG is added to an immunoglobulin molecule by enzymatically oxidizing a glycan on the immunoglobulin and then contacting the glycan with an amino-PEG molecule.

5 In each of the methods described above, poly(ethylene glycol) is added in a random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivatization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product.

Two principal classes of enzymes are used in the synthesis of carbohydrates,  
10 glycosyltransferases (e.g., sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (e.g.,  $\beta$ -mannosidase,  $\beta$ -glucosidase), and endoglycosidases (e.g., Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, see, Crout et al., *Curr. Opin. Chem. Biol.* 2: 98-  
15 111 (1998).

Glycosyltransferases modify the oligosaccharide structures on peptides. Glycosyltransferases are effective for producing specific products with good stereochemical and regiochemical control. Glycosyltransferases have been used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on peptides  
20 produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example,  $\beta$ -1,4-galactosyltransferase is used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of carbohydrates (see, e.g.,  
25 Wong et al., *J. Org. Chem.* 47: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of  $\alpha$ -sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (see, e.g., Kevin et al., *Chem. Eur. J.* 2: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific hydroxyl of a  
30 saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase

(Ichikawa *et al.*, *J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use *see*, Koeller *et al.*, *Nature Biotechnology* **18**: 835-841 (2000). *See also*, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

5        Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. However, under appropriate conditions, they can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate. The glycosidase binds a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water  
10 to yield the hydrolysis product, or by an acceptor, to generate a new glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the  $\beta$ -mannoside linkage, which is formed by the action of  $\beta$ -mannosidase (Singh *et al.*, *Chem. Commun.* 993-994 (1996)).

      In another exemplary application of the use of a glycosidase to form a glycosidic  
15 linkage, a mutant glycosidase has been prepared in which the normal nucleophilic amino acid within the active site is changed to a non-nucleophilic amino acid. The mutant enzyme does not hydrolyze glycosidic linkages, but can still form them. Such a mutant glycosidase is used to prepare oligosaccharides using an  $\alpha$ -glycosyl fluoride donor and a glycoside acceptor molecule (Withers *et al.*, U.S. Patent No. 5,716,812).

20        Although their use is less common than that of the exoglycosidases, endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*- $\beta$ -N-acetylglucosamines such as *endo*-F, *endo*-M (Wang *et al.*, *Tetrahedron Lett.* **37**: 1975-1978); and Haneda *et al.*,  
25 *Carbohydr. Res.* **292**: 61-70 (1996)).

      In addition to their use in preparing carbohydrates, the enzymes discussed above are applied to the synthesis of glycopeptides as well. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. *et al.*, *J. Am. Chem. Soc.* **119**: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide  
30 with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc

residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of  $\beta$ -1,4-galactosyltransferase,  $\alpha$ -2,3-sialyltransferase and  $\alpha$ -1,3-fucosyltransferase V. However, while each enzymatically catalyzed step proceeded in excellent yield, such procedures have not been adapted for the generation of glycopeptides on an industrial scale.

Methods combining both chemical and enzymatic synthetic elements are also known in the art. For example, Yamamoto and coworkers (*Carbohydr. Res.* **305**: 415-422 (1998)) reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglycosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of human transferrin peptide. The saccharide portion was added to the peptide by treating it with an endo- $\beta$ -N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

The use of glycosyltransferases to modify peptide structure with reporter groups has been explored. For example, Brossmer *et al.* (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent-labeling of cell surfaces, glycoproteins and peptides. Gross *et al.* (*Analyt. Biochem.* **186**: 127 (1990)) describe a similar assay. Bean *et al.* (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation of a deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays for the presence of glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid has not been disclosed or suggested in the prior art.

Considerable effort has also been directed towards the modification of cell surfaces by altering glycosyl residues presented by those surfaces. For example, Fukuda and coworkers have developed a method for attaching glycosides of defined structure onto cell surfaces. The method exploits the relaxed substrate specificity of a fucosyltransferase that can transfer  
5 fucose and fucose analogs bearing diverse glycosyl substrates (Tsuboi *et al.*, *J. Biol. Chem.* 271: 27213 (1996)).

Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the  
10 corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing modifying group. For example, Casares *et al.* (*Nature Biotech.* 19: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

Glycosyl residues have also been modified to contain ketone groups. For example, Mahal and co-workers (*Science* 276: 1125 (1997)) have prepared N-levulinoyl mannosamine  
15 ("ManLev"), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. See, also Saxton *et al.*, *Science* 287: 2007 (2000); Hang *et al.*, *J. Am. Chem. Soc.* 123: 1242 (2001); Yarema *et al.*, *J. Biol. Chem.* 273: 31168 (1998); and Charter *et al.*, *Glycobiology* 10: 1049 (2000).

20 The methods of modifying cell surfaces have not been applied in the absence of a cell to modify a glycosylated or non-glycosylated peptide. Further, the methods of cell surface modification are not utilized for the enzymatic incorporation preformed modified glycosyl donor moiety into a peptide. Moreover, none of the cell surface modification methods are practical for producing glycosyl-modified peptides on an industrial scale.

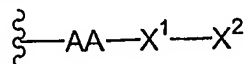
25 Despite the efforts directed toward the enzymatic elaboration of saccharide structures, there remains still a need for an industrially practical method for the modification of glycosylated and non-glycosylated peptides with modifying groups such as water-soluble polymers, therapeutic moieties, biomolecules and the like. Of particular interest are methods in which the modified peptide has improved properties, which enhance its use as a  
30 therapeutic or diagnostic agent. The present invention fulfills these and other needs.

## SUMMARY OF THE INVENTION

The invention includes a multitude of methods of remodeling a peptide to have a specific glycan structure attached thereto. Although specific glycan structures are described herein, the invention should not be construed to be limited to any one particular structure. In addition, although specific peptides are described herein, the invention should not be limited by the nature of the peptide described, but rather should encompass any and all suitable peptides and variations thereof.

The description which follows discloses the preferred embodiments of the invention and provides a written description of the claims appended hereto. The invention encompasses any and all variations of these embodiments that are or become apparent following a reading of the present specification.

The invention includes a cell-free, in vitro method of remodeling an erythropoietin (EPO) peptide, the peptide having the formula:



wherein

AA is a terminal or internal amino acid residue of the peptide;

X<sup>1</sup>-X<sup>2</sup> is a saccharide covalently linked to the AA, wherein

X<sup>1</sup> is a first glycosyl residue; and

X<sup>2</sup> is a second glycosyl residue covalently linked to X<sup>1</sup>, wherein X<sup>1</sup> and X<sup>2</sup> are

selected from monosaccharyl and oligosaccharyl residues;

the method comprising:

(a) removing X<sup>2</sup> or a saccharyl subunit thereof from the peptide, thereby forming a truncated glycan; and

(b) contacting the truncated glycan with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the truncated glycan, thereby remodeling the EPO peptide.



The method further comprises:

- (c) removing  $X^1$ , thereby exposing the AA; and
- (d) contacting the AA with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the AA, thereby remodeling the EPO peptide.

5

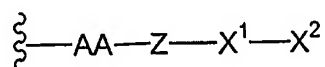
The method additionally comprises:

- (e) prior to step (b), removing a group added to the saccharide during post-translational modification.

In one aspect, the group is a member selected from phosphate, sulfate, carboxylate and esters thereof.

10

In one aspect of the method, the peptide has the formula:



wherein

Z is a member selected from O, S, NH and a crosslinker.

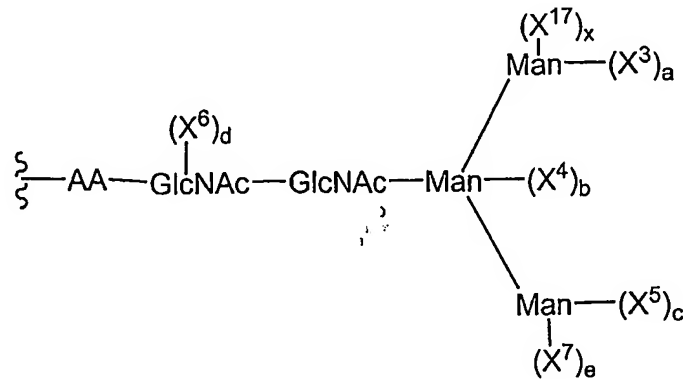
In one embodiment, at least one of the glycosyl donors comprises a modifying group.

15

In this and in other embodiments, the modifying group is a member selected from the group consisting of a polymer, a therapeutic moiety, a detectable label, a reactive linker group, a targeting moiety, and a peptide. Preferably, the modifying group is a water soluble polymer, preferably, poly(ethylene glycol), wherein preferably, the poly(ethylene glycol) has a molecular weight distribution that is essentially homodisperse.

20

There is also provided a cell-free in vitro method of remodeling an EPO peptide, the peptide having the formula:



wherein

X<sup>3</sup>, X<sup>4</sup>, X<sup>5</sup>, X<sup>6</sup>, X<sup>7</sup>, and X<sup>17</sup> are independently selected monosaccharyl or oligosaccharyl residues; and

5           a, b, c, d, e and x are independently selected from the integers 0, 1 and 2, with  
the proviso that at least one member selected from a, b, c, d, and e and  
x are 1 or 2; the method comprising:

(a) removing at least one of X<sup>3</sup>, X<sup>4</sup>, X<sup>5</sup>, X<sup>6</sup>, X<sup>7</sup>, or X<sup>17</sup>, a saccharyl subunit thereof from the peptide, thereby forming a truncated glycan; and

10 (b) contacting the truncated glycan with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the truncated glycan, thereby remodeling the EPO peptide.

In one embodiment, the removing of step (a) produces a truncated glycan in  
15 which a, b, c, e and x are each 0.

In another embodiment,  $X^3$ ,  $X^5$ , and  $X^7$ , are selected from the group consisting of (mannose)<sub>z</sub> and (mannose)<sub>z</sub>-(X<sup>8</sup>)<sub>y</sub>

wherein

20 X<sup>8</sup> is a glycosyl moiety selected from mono- and oligo-saccharides;  
y is an integer selected from 0 and 1; and

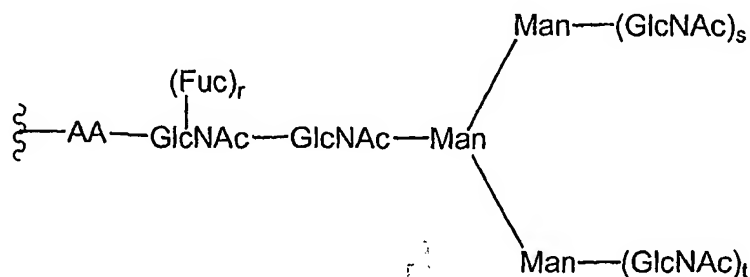
z is an integer between 1 and 20, wherein  
when z is 3 or greater, (mannose)<sub>z</sub> is selected from linear and branched  
structures.

In a further embodiment,  $X^4$  is selected from the group consisting of GlcNAc  
5 and xylose.

In yet another embodiment,  $X^3$ ,  $X^5$ , and  $X^7$  are (mannose)<sub>u</sub>, wherein u is selected from the integers between 1 and 20, and when u is 3 or greater, (mannose)<sub>u</sub> is selected from linear and branched structures.

In one aspect, at least one of the glycosyl donors comprises a modifying  
10 group.

There is further provided a cell-free in vitro method of remodeling an EPO peptide comprising a glycan having the formula:



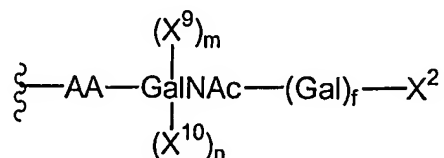
wherein

1.5 r, s, and t are integers independently selected from 0 and 1,  
the method comprising:

(a) contacting the peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the glycan, thereby remodeling the EPO peptide.

In one aspect, at least one of the glycosyl donors comprises a modifying group.

In another aspect, the peptide has the formula:

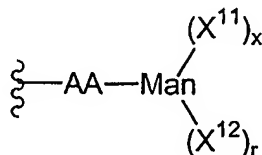


5 wherein

$X^9$  and  $X^{10}$  are independently selected monosaccharyl or oligosaccharyl residues; and

$m$ ,  $n$  and  $f$  are integers selected from 0 and 1.

Further, the peptide has the formula:



10

wherein

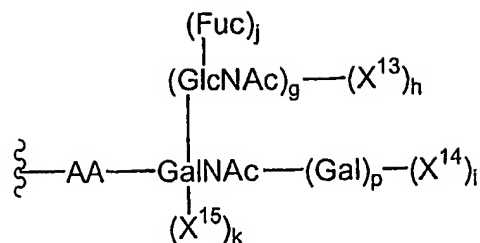
$X^{11}$  and  $X^{12}$  are independently selected glycosyl moieties; and

$r$  and  $x$  are integers independently selected from 0 and 1.

In another aspect,  $X^{11}$  and  $X^{12}$  are (mannose)<sub>q</sub>, wherein

15  $q$  is selected from the integers between 1 and 20, and when  $q$  is three or greater, (mannose)<sub>q</sub> is selected from linear and branched structures.

In addition, the peptide has the formula:



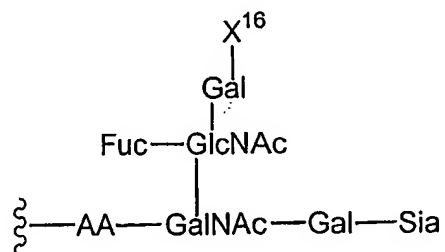
wherein

$\text{X}^{13}$ ,  $\text{X}^{14}$ , and  $\text{X}^{15}$  are independently selected glycosyl residues; and  
 $g$ ,  $h$ ,  $i$ ,  $j$ ,  $k$ , and  $p$  are independently selected from the integers 0 and 1, with  
 the proviso that at least one of  $g$ ,  $h$ ,  $i$ ,  $j$ ,  $k$  and  $p$  is 1.

The method also includes wherein

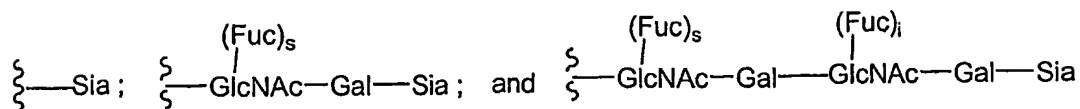
$\text{X}^{14}$  and  $\text{X}^{15}$  are members independently selected from GlcNAc and Sia; and  $i$   
 and  $k$  are independently selected from the integers 0 and 1, with the  
 proviso that at least one of  $i$  and  $k$  is 1 and if  $k$  is 1,  $g$ ,  $h$  and  $j$  are 0.

In an additional aspect, the peptide has the formula:



wherein

$\text{X}^{16}$  is a member selected from:

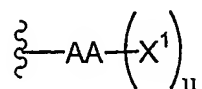


wherein

$s$  and  $i$  are integers independently selected from 0 and 1.

In one embodiment, the removing utilizes a glycosidase.

Also included is a cell-free, in vitro method of remodeling an EPO peptide having the formula:



5 wherein

AA is a terminal or internal amino acid residue of the peptide;

X<sup>1</sup> is a glycosyl residue covalently linked to the AA, selected from monosaccharyl and oligosaccharyl residues; and

u is an integer selected from 0 and 1,

10 the method comprising:

contacting the peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the truncated glycan, wherein the glycosyl donor comprises a modifying group, thereby remodeling the EPO peptide.

15 The invention further provides a covalent conjugate between an EPO peptide and a modifying group that alters a property of the peptide, wherein the modifying group is covalently attached to the peptide at a preselected glycosyl or amino acid residue of the peptide via an intact glycosyl linking group.

20 In one aspect, the modifying group is a member selected from the group consisting of a polymer, a therapeutic moiety, a detectable label, a reactive linker group, a targeting moiety, and a peptide. In another aspect, the modifying group and an intact glycosyl linking group precursor are linked as a covalently attached unit to the peptide via the action of an enzyme, the enzyme converting the precursor to the intact glycosyl linking group, thereby forming the conjugate. In a further aspect, the covalent conjugate comprises a

first modifying group covalently linked to a first residue of the peptide via a first intact glycosyl linking group, and a second glycosyl linking group linked to a second residue of the peptide via a second intact glycosyl linking group. In one embodiment, the first residue and the second residue are structurally identical. In another embodiment, the first residue and the second residue have different structures. In an additional embodiment, the first residue and the second residue are glycosyl residues. Further, the first residue and the second residue are amino acid residues. Also, the peptide may be remodeled prior to forming the conjugate. In another aspect, the remodeled peptide is remodeled to introduce an acceptor moiety for the intact glycosyl linking group. In yet a further aspect, the modifying group is a water-soluble polymer. In one aspect, the intact glycosyl linking unit is a member selected from the group consisting of a sialic acid residue, a Gal residue, a GlcNAc residue, and a GalNAc residue.

There is also provided a method of forming a covalent conjugate between a polymer and a glycosylated or non-glycosylated peptide, wherein the polymer is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide and the polymer, the method comprising:

contacting the peptide with a mixture comprising a nucleotide sugar covalently linked to the polymer and a glycosyltransferase for which the nucleotide sugar is a substrate under conditions sufficient to form the conjugate, wherein the peptide is EPO.

In one aspect, the polymer is a water-soluble polymer. In another aspect, the glycosyl linking group is covalently attached to a glycosyl residue covalently attached to the peptide. In yet a further aspect, the glycosyl linking group is covalently attached to an amino acid residue of the peptide. In an additional aspect, the polymer comprises a member selected from the group consisting of a polyalkylene oxide and a polypeptide. In another aspect, the polyalkylene oxide is poly(ethylene glycol).

In yet a further aspect, the glycosyltransferase is selected from the group consisting of sialyltransferase, galactosyltransferase, glucosyltransferase, GalNAc

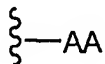
transferase, and a GlcNAc transferase. Additionally, the glycosyltransferase is recombinantly produced and may be either a recombinant prokaryotic or eukaryotic enzyme.

In one embodiment, the nucleotide sugar is selected from the group consisting of UDP-glycoside, CMP-glycoside, and GDP-glycoside. Further, the nucleotide sugar is selected from the group consisting of UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, UDP-N-acetylgalactosamine, UDP-N-acetylglucosamine, GDP-mannose, GDP-fucose, CMP-sialic acid, CMP-NeuAc. In addition, the glycosylated peptide is partially deglycosylated prior to the contacting. Further, the intact glycosyl linking group is a sialic acid residue. In addition, the method is performed in a cell-free environment. Also, the covalent conjugate is isolated, preferably by membrane filtration.

The invention further provides a composition for forming a conjugate between a peptide and a modified sugar, the composition comprising: an admixture of a modified sugar, a glycosyltransferase, and a peptide acceptor substrate, wherein the modified sugar has covalently attached thereto a member selected from a polymer, a therapeutic moiety and a biomolecule, wherein the peptide is EPO.

In addition, there is provided an EPO peptide remodeled by the methods of the invention and pharmaceutical compositions comprising such EPO peptides.

Also provided is a cell-free, in vitro method of remodeling a peptide having the formula:



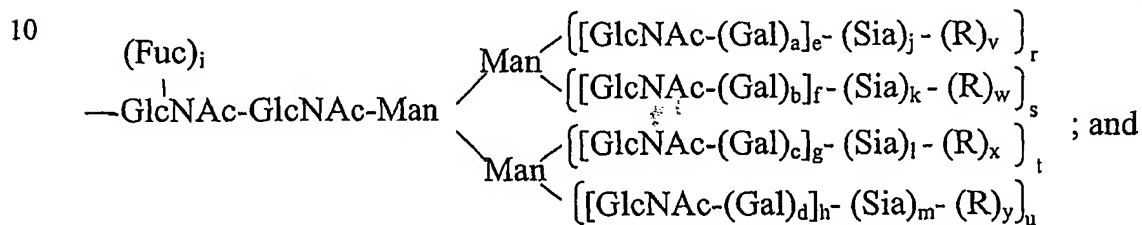
wherein

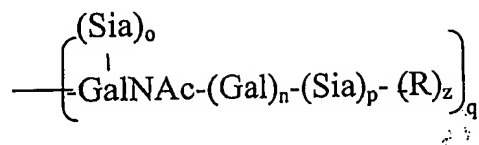
AA is a terminal or internal amino acid residue of the peptide,  
the method comprising:



contacting the peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the amino acid residue, wherein the glycosyl donor comprises a modifying group, thereby remodeling the peptide, wherein the peptide is EPO.

- 5 In addition, there is provided a method for forming a conjugate between an EPO peptide and a modifying group, wherein the modifying group is covalently attached to the EPO peptide through an intact glycosyl linking group, the EPO peptide comprising a glycosyl residue having a formula which is a member selected from:





5 wherein

a, b, c, d, i, n, o, p, q, r, s, t, and u are members independently selected from 0 and 1;

e, f, g, and h are members independently selected from the integers between 0 and 4;

10 j, k, l, and m are members independently selected from the integers between 0 and 20;

v, w, x, y, and z are 0; and

R is a modifying group, a mannose or an oligomannose;

the method comprising:

15 (a) contacting the EPO peptide with a glycosyltransferase and a modified glycosyl donor, comprising a glycosyl moiety which is a substrate for the glycosyltransferase covalently linked to the modifying group, under conditions appropriate for the formation of the intact glycosyl linking group.

20 The method further comprises:

(b) prior to step (a), contacting the EPO peptide with a sialidase under conditions appropriate to remove sialic acid from the EPO peptide.

The method additionally comprises:

25 (c) contacting the product of step (a) with a sialyltransferase and a sialic acid donor under conditions appropriate to transfer sialic acid to the product.

The method further comprises:

(d) prior to step (a), contacting the EPO peptide with a galactosidase operating synthetically under conditions appropriate to add a galactose to the EPO peptide.

In addition, the method comprises:

- (e) prior to step (a), contacting the EPO peptide with a galactosyl transferase and a galactose donor under conditions appropriate to transfer the galactose to the EPO peptide.

5

The method also comprises:

- (f) contacting the product from step (e) with ST3Gal3 and a sialic acid donor under conditions appropriate to transfer sialic acid to the product.

The method also comprises:

10

- (g) contacting the product from step (a) with a moiety that reacts with the modifying group, thereby forming a conjugate between the intact glycosyl linking group and the moiety.

In addition, the method comprises:

15

- (h) prior to step (a), contacting the EPO peptide with N-acetylglucosamine transferase and a GlcNAc donor under conditions appropriate to transfer GlcNAc to the EPO peptide.

In one aspect, the modifying group is a member selected from a polymer, a toxin, a radioisotope, a therapeutic moiety and a glycoconjugate.

In another aspect,

20

- a, b, c, d, e, f, g, n, and q are 1;  
h is a member selected from the integers between 1 and 3;  
i, j, k, l, m, o, p, r, s, t, and u are members independently selected from 0 and 1; and  
v, w, x, y and z are 0.

In yet another aspect,

25

- a, b, c, d, f, h, j, k, l, m, q, s, u, v, w, x, y, and z are 0; and  
e, g, i, r, and t are members independently selected from 0 and 1.

In an additional aspect,

a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, and u are members independently selected from 0 and 1; and  
v, w, x, y, and z are 0.

5 In a further aspect,  
a, b, c, d, e, f, g, n, and q are 1;  
h is a member selected from the integers between 1 and 3;  
i, j, k, l, m, o, p, r, s, t, and u are members independently selected from 0 and 1; and  
v, w, x, y and z are 0.

10 In another aspect,  
a, b, c, d, f, h, j, k, l, m, o, p, s, u, v, w, x, y, and z are 0; and  
e, g, i, n, q, r, and t are independently selected from 0 and 1.

Additionally,  
a, b, c, d, f, h, j, k, l, m, n, o, p, s, u, v, w, x, y, and z are 0; and  
e, g, i, q, r, and t are members independently selected from 0 and 1.

15 Further  
q is 1;  
a, b, c, d, e, f, g, h, i, n, r, s, t, and u are members independently selected from 0 and 1;  
and  
j, k, l, m, o, p, v, w, x, y, and z are 0.

20 There is also provided an EPO peptide conjugate formed by the methods of the invention.

25 Further provided is an EPO peptide comprising one or more glycans, having a glycoconjugate molecule covalently attached to the peptide. In one aspect, the one or more glycans is a monoantennary glycan. In another aspect, the one or more glycans is a biantennary glycan. In a further aspect, the one or more glycans is a triantennary glycan. In

yet another aspect, the one or more glycans is at least a triantennary glycan. In a further aspect, the one or more glycans comprises at least two glycans comprising a mixture of mono or multiantennary glycans. In yet another aspect, the one or more glycans is selected from an N-linked glycan and an O-linked glycan. In a further aspect, the one or more glycans is at least two glycans selected from an N-linked and an O-linked glycan. Additionally, the peptide is expressed in a cell selected from the group consisting of a prokaryotic cell and a eukaryotic cell, and the eukaryotic cell is selected from the group consisting of a mammalian cell, an insect cell and a yeast cell.

Also provided in the invention is a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase. In one aspect, the glycoPEGylated EPO peptide comprises at least one mono-antennary glycan. In another aspect, all of the glycans are N-linked and are mono-antennary. In a further aspect, all of the glycans are N-linked and at least one of the glycans comprise the poly(ethylene glycol). In an additional aspect, more than one of the glycans comprises the poly(ethylene glycol). In a further aspect, all of the glycans are N-linked and all of the glycans comprise the poly(ethylene glycol). Additionally, the glycoPEGylated peptide comprises at least three mono-antennary glycans having the poly(ethylene glycol) covalently attached thereto.

20

There is further provided a glycoPEGylated EPO peptide, wherein the EPO peptide comprises three or more glycans. In one aspect, at least one of the glycans comprises the poly(ethylene glycol) covalently attached thereto. In another aspect, more than one of the glycans comprises the poly(ethylene glycol) covalently attached thereto. In a further aspect, all of the glycans comprise the poly(ethylene glycol) covalently attached thereto. Additionally, the poly(ethylene glycol) is linked to at least one sugar moiety selected from the group consisting of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal) and a sialic acid (SA). Additionally, the sialic acid is N-acetylneuraminic acid. Further, the EPO peptide does not comprise an O-linked glycan. In addition, the EPO peptide comprises at least one O-linked glycan. Further, the O-linked peptide comprises the poly(ethylene

30

glycol) covalently attached thereto. In addition, the EPO peptide is recombinantly expressed in a cell. Further, the cell is selected from the group consisting of an insect cell, a yeast cell and a mammalian cell. Preferably, the mammalian cell is a CHO cell. In one aspect, the poly(ethylene glycol) has a molecular weight selected from the group consisting of about 1 kDa, 2 kDa, 5 kDa, 10 kDa, 20 kDa, 30 kDa and 40 kDa. In another aspect, the EPO peptide is selected from the group consisting of a naturally occurring EPO peptide and a mutated EPO peptide. In another aspect, mutated EPO peptide comprises the amino acid sequence of SEQ ID NO:73 having at least one mutation selected from the group consisting of Arg<sup>139</sup> to Ala<sup>139</sup>, Arg<sup>143</sup> to Ala<sup>143</sup> and Lys<sup>154</sup> to Ala<sup>154</sup>.

There is also provided a method of making a glycoPEGylated EPO peptide, the method comprising the steps of:

(a) contacting an EPO peptide with a mixture comprising a nucleotide sugar covalently linked to poly(ethylene glycol) and a glycosyltransferase under conditions sufficient to transfer the poly(ethylene glycol) to the EPO peptide.

In one embodiment, the sugar of the nucleotide sugar is selected from the group consisting of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal) and a sialic acid (SA). In another embodiment, the sialic acid is N-acetylneuraminic acid (NAN). In a further embodiment, the poly(ethylene glycol) has a molecular weight selected from the group consisting of about 1 kDa, 2 kDa, 5 kDa, 10 kDa, 20 kDa, 30 kDa and 40 kDa. In an additional embodiment, the EPO peptide is recombinantly expressed in a cell, where the cell may be selected from the group consisting of an insect cell, a yeast cell and a mammalian cell. In one aspect, the EPO peptide is selected from the group consisting of a naturally occurring EPO peptide and a mutated EPO peptide. In another aspect, the mature EPO has the sequence of SEQ ID NO:73. In a further aspect, the mutated EPO peptide comprises the amino acid sequence of SEQ ID NO: 73 having at least one mutation selected from the group consisting of Arg<sup>139</sup> to Ala<sup>139</sup>, Arg<sup>143</sup> to Ala<sup>143</sup> and Lys<sup>154</sup> to Ala<sup>154</sup>.

In another aspect of the method of the invention, before step (a): the method comprises:

(b) contacting the EPO peptide with a mixture comprising a nucleotide-N-acetylglucosamine (GlcNAc) molecule and an N-acetylglucosamine transferase (GnT) for which the nucleotide-GlcNAc is a substrate under conditions sufficient to form a bond between the GlcNAc and the EPO, wherein the GnT is selected from the group consisting of  
5 GnT I, GnT II, GnT III, GnT IV, GnT V and GnT VI.

In one aspect, the mixture comprises one GnT selected from the group consisting of GnT I, GnT II, GnT IV, GnT V and GnT VI. In another aspect, the glycoPEGylated EPO peptide comprises at least one mono-antennary glycan. In a further  
10 aspect, the sugar of the nucleotide sugar is galactose and the glycosyltransferase is galactosyl transferase I (GalT I).

In an additional aspect of the method, before step (a) but after step (b):

(c) contacting the EPO peptide with a mixture comprising a nucleotide galactose (Gal)  
15 and galactosyl transferase I (GalT I) under conditions sufficient to transfer galactose to the EPO peptide.

In one embodiment, in step (a), the sugar of the nucleotide sugar is sialic acid and the glycosyltransferase is a sialyltransferase. In another embodiment, the sialic acid is N-acetylneuraminic acid (NAN). In a further embodiment, the sialyltransferase is selected from  
20 the group consisting of  $\alpha(2,3)$ sialyltransferase,  $\alpha(2,6)$ sialyltransferase and (2,8)sialyltransferase.

There is also provided a glycoPEGylated EPO, the EPO comprising the sequence of SEQ ID NO:73. In one aspect, the EPO comprises the sequence of SEQ ID  
25 NO:73 and further comprising a mutation in the sequence.

There is further provided in the invention a method of making a glycoPEGylated EPO peptide, the method comprising the steps of:

(a) contacting an EPO peptide with a mixture comprising a nucleotide sugar  
30 covalently linked to poly(ethylene glycol) and a glycosyltransferase under conditions

sufficient to transfer the poly(ethylene glycol) to the EPO peptide, wherein the glycosyltransferase is a fucosyltransferase.

In one aspect, the fucosyltransferase is selected from the group consisting of  
5 fucosyltransferase I, fucosyltransferase III, fucosyltransferase IV, fucosyltransferase V, fucosyltransferase VI and fucosyltransferase VII, and in another, EPO is expressed in a CHO cell.

There is further provided a method of treating a mammal having anemia, the  
10 method comprising administering to the mammal an EPO peptide having one or more glycans having a glycoconjugate molecule attached to the peptide, wherein the EPO is administered in an amount effective to increase the hematocrit level in the mammal. In this and in other embodiments, the mammal is a human.

Also provided is a method of providing erythropoietin therapy to a mammal,  
15 the method comprising administering an effective amount of a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase, wherein the EPO is administered in an amount  
20 effective to increase the hematocrit level in the mammal.

In addition, there is provided a method of treating a mammal having anemia, the method comprising administering to the mammal a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol)  
25 molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase, wherein the EPO is administered in an amount effective to increase the hematocrit level in the mammal. In one aspect, the anemia is associated with chemotherapy.



Further provided is a method of treating a kidney dialysis patient, the method comprising administering to the patient a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase, wherein the EPO is administered in an amount effective to increase the hematocrit level in the patient.

## BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1 is a scheme depicting a trimannosyl core glycan (left side) and the enzymatic process for the generation of a glycan having a bisecting GlcNAc (right side).

Figure 2 is a scheme depicting an elemental trimannosyl core structure and complex chains in various degrees of completion. The *in vitro* enzymatic generation of an elemental trimannosyl core structure from a complex carbohydrate glycan structure which does not contain a bisecting GlcNAc residue is shown, as is the generation of a glycan structure therefrom which contains a bisecting GlcNAc. Symbols: squares: GlcNAc; light circles: Man; dark circles: Gal; triangles: NeuAc.

Figure 3 is a scheme for the enzymatic generation of a sialylated glycan structure (right side) beginning with a glycan having a trimannosyl core and a bisecting GlcNAc (left side).

Figure 4 is a scheme of a typical high mannose containing glycan structure (left side) and the enzymatic process for reduction of this structure to an elemental trimannosyl core structure. In this scheme, X is mannose as a monosaccharide, an oligosaccharide or a polysaccharide.

Figure 5 is a diagram of a fucose and xylose containing N-linked glycan structure typically produced in plant cells.

Figure 6 is a diagram of a fucose containing N-linked glycan structure typically produced in insect cells. Note that the glycan may have no core fucose, it may have a single core fucose with either linkage, or it may have a single core fucose having a preponderance of one linkage.

Figure 7 is a scheme depicting a variety of pathways for the trimming of a high mannose structure and the synthesis of complex sugar chains therefrom. Symbols: squares: GlcNAc; circles: Man; diamonds: fucose; pentagon: xylose.

Figure 8 is a scheme depicting *in vitro* strategies for the synthesis of complex structures from an elemental trimannosyl core structure. Symbols: Squares: GlcNAc; light circles: Man; dark circles: Gal; dark triangles: NeuAc; GnT: N-acetyl glucosaminyltransferase; GalT: galactosyltransferase; ST: sialyltransferase.

Figure 9 is a scheme depicting two *in vitro* strategies for the synthesis of monoantennary glycans, and the optional glycoPEGylation of the same. Dark squares: GlcNAc; dark circles: Man; light circles: Gal; dark triangles: sialic acid.

Figure 10 is a scheme depicting two *in vitro* strategies for the synthesis of monoantennary glycans, and the optional glycoPEGylation of the same. Dark squares: GlcNAc; dark circles: Man; light circles: Gal; dark triangles: sialic acid.

Figure 11 is a scheme depicting various complex structures, which may be synthesized from an elemental trimannosyl core structure. Symbols: Squares: GlcNAc; light circles: Man; dark circles: Gal; triangles: NeuAc; diamonds: fucose; FT and FucT: fucosyltransferase; GalT: galactosyltransferase; ST: sialyltransferase; Le: Lewis antigen; SLe: sialylated Lewis antigen.

Figure 12 is an exemplary scheme for preparing O-linked glycopeptides originating with serine or threonine. Optionally, a water soluble polymer (WSP) such as poly(ethylene glycol) is added to the final glycan structure.

Figure 13 is a series of diagrams depicting the four types of O-glycan structures, termed cores 1 through 4. The core structure is outlined in dotted lines.

Figure 14, comprising Figure 14A and Figure 14B, is a series of schemes showing an exemplary embodiment of the invention in which carbohydrate residues comprising complex

carbohydrate structures and/or high mannose high mannose structures are trimmed back to the first generation biantennary structure. Optionally, fucose is added only after reaction with GnT I. A modified sugar bearing a water-soluble polymer (WSP) is then conjugated to one or more of the sugar residues exposed by the trimming back process.

5           Figure 15 is a scheme similar to that shown in Figure 4, in which a high mannose or complex structure is "trimmed back" to the mannose beta-linked core and a modified sugar bearing a water soluble polymer is then conjugated to one or more of the sugar residues exposed by the trimming back process. Sugars are added sequentially using glycosyltransferases.

10           Figure 16 is a scheme similar to that shown in Figure 4, in which a high mannose or complex structure is trimmed back to the GlcNAc to which the first mannose is attached, and a modified sugar bearing a water soluble polymer is then conjugated to one or more of the sugar residues exposed by the trimming back process. Sugars are added sequentially using glycosyltransferases.

15           Figure 17 is a scheme similar to that shown in Figure 4, in which a high mannose or complex structure is trimmed back to the first GlcNAc attached to the Asn of the peptide, following which a water soluble polymer is conjugated to one or more sugar residues which have subsequently been added on. Sugars are added sequentially using glycosyltransferases.

20           Figure 18, comprising Figure 18A and 18B, is a scheme in which an N-linked carbohydrate is optionally trimmed back from a high mannose or complex structure, and subsequently derivatized with a modified sugar moiety (Gal or GlcNAc) bearing a water-soluble polymer.

25           Figure 19, comprising Figure 19A and 19B, is a scheme in which an N-linked carbohydrate is trimmed back from a high mannose or complex structure and subsequently derivatized with a sialic acid moiety bearing a water-soluble polymer. Sugars are added sequentially using glycosyltransferases.

30           Figure 20 is a scheme in which an N-linked carbohydrate is optionally trimmed back from a high mannose or complex structure and subsequently derivatized with one or more sialic acid moieties, and terminated with a sialic acid derivatized with a water-soluble polymer. Sugars are added sequentially using glycosyltransferases.

Figure 21 is a scheme in which an O-linked saccharide is "trimmed back" and subsequently conjugated to a modified sugar bearing a water-soluble polymer. In the exemplary scheme, the carbohydrate moiety is "trimmed back" to the first generation of the biantennary structure.

5        Figure 22 is an exemplary scheme for trimming back the carbohydrate moiety of an O-linked glycopeptide to produce a mannose available for conjugation with a modified sugar having a water-soluble polymer attached thereto.

Figure 23, comprising Figure 23A to Figure 23C, is a series of exemplary schemes. Figure 23A is a scheme that illustrates addition of a PEGylated sugar, followed by the  
10       addition of a non-modified sugar. Figure 23B is a scheme that illustrates the addition of more than one kind of modified sugar onto one glycan. Figure 23C is a scheme that illustrates the addition of different modified sugars onto O-linked glycans and N-linked glycans.

Figure 24 is a diagram of various methods of improving the therapeutic function of a peptide by glycan remodeling, including conjugation.

15       Figure 25 is a set of schemes for glycan remodeling of a therapeutic peptide to treat Gaucher Disease.

Figure 26 is a scheme for glycan remodeling to generate glycans having a terminal mannose-6-phosphate moiety.

20       Figure 27 is a diagram illustrating the array of glycan structures found on CHO-produced glucocerebrosidase (Cerezyme™) after sialylation.

Figure 28, comprising Figure 28A to Figure 28Z and Figure 28AA to Figure 28CC, is a list of peptides useful in the methods of the invention.

Figure 29, comprising Figures 29A to 29G, provides exemplary schemes for remodeling glycan structures on granulocyte colony stimulating factor (G-CSF). Figure 29A  
25       is a diagram depicting the G-CSF peptide indicating the amino acid residue to which a glycan is bonded, and an exemplary glycan formula linked thereto. Figure 29B to 29G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 29A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 30, comprising Figures 30A to 30EE sets forth exemplary schemes for  
30       remodeling glycan structures on interferon-alpha. Figure 30A is a diagram depicting the interferon-alpha isoform 14c peptide indicating the amino acid residue to which a glycan is

bonded, and an exemplary glycan formula linked thereto. Figure 30B to 30D are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 30A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30E is a diagram depicting the interferon-alpha isoform 14c peptide indicating the amino acid

5 residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 30F to 30N are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 30E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30O is a diagram depicting the interferon-alpha isoform 2a or 2b peptides indicating the amino acid residue to which a glycan is linked, and an exemplary

10 glycan formula linked thereto. Figure 30P to 30W are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 30O based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30X is a diagram depicting the interferon-alpha-mucin fusion peptides indicating the residue(s) which is linked to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure

15 30Y to 30AA are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 30X based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30BB is a diagram depicting the interferon-alpha-mucin fusion peptides and interferon-alpha peptides indicating the residue(s) which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 30CC to 30EE are  
20 diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 30BB based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 31, comprising Figures 31A to 31S, sets forth exemplary schemes for remodeling glycan structures on interferon-beta. Figure 31A is a diagram depicting the  
25 interferon-beta peptide indicating the amino acid residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 31B to 31O are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 31A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 31P is a diagram depicting the interferon-beta peptide indicating the amino acid residue to which a glycan is  
30 linked, and an exemplary glycan formula linked thereto. Figure 31Q to 31S are diagrams of

contemplated remodeling steps of the glycan of the peptide in Figure 31P based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 32, comprising Figures 32A to 32D, sets forth exemplary schemes for remodeling glycan structures on Factor VII and Factor VIIa. Figure 32A is a diagram depicting the Factor-VII and Factor-VIIa peptides A (solid line) and B (dotted line) indicating the residues which bind to glycans contemplated for remodeling, and the formulas for the glycans. Figure 32B to 32D are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 32A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 33, comprising Figures 33A to 33G, sets forth exemplary schemes for remodeling glycan structures on Factor IX. Figure 33A is a diagram depicting the Factor-IX peptide indicating residues which bind to glycans contemplated for remodeling, and formulas of the glycans. Figure 33B to 33G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 33A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 34, comprising Figures 34A to 34J, sets forth exemplary schemes for remodeling glycan structures on follicle stimulating hormone (FSH), comprising  $\alpha$  and  $\beta$  subunits. Figure 34A is a diagram depicting the Follicle Stimulating Hormone peptides FSH $\alpha$  and FSH $\beta$  indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 34B to 34J are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 34A based on the type of cell the peptides are expressed in and the desired remodeled glycan structures.

Figure 35, comprising Figures 35A to 35AA, sets forth exemplary schemes for remodeling glycan structures on Erythropoietin (EPO). Figure 35A is a diagram depicting the EPO peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 35B to 35S are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 35A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 35T is a diagram depicting the EPO peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 35U to 35W are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 35T based on the type of cell the peptide is

expressed in and the desired remodeled glycan structure. Figure 35X is a diagram depicting the EPO peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 35Y to 35AA are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 35X based on the type of cell the peptide is

5 expressed in and the desired remodeled glycan structure.

Figure 36, comprising Figures 36A to 36K, sets forth exemplary schemes for remodeling glycan structures on Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). Figure 36A is a diagram depicting the GM-CSF peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 36B to 36G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 36A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 36H is a diagram depicting the GM-CSF peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 36I to 36K are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 36H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 37, comprising Figures 37A to 37N, sets forth exemplary schemes for remodeling glycan structures on interferon-gamma. Figure 37A is a diagram depicting an interferon-gamma peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 37B to 37G are diagrams of contemplated remodeling steps of the peptide in Figure 37A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 37H is a diagram depicting an interferon-gamma peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 37I to 37N are diagrams of contemplated remodeling steps of the peptide in Figure 37H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 38, comprising Figures 38A to 38N, sets forth exemplary schemes for remodeling glycan structures on  $\alpha_1$ -antitrypsin (ATT, or  $\alpha_1$  protease inhibitor). Figure 38A is a diagram depicting an AAT peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 38B to 38F are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure

38A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 38G is a diagram depicting an AAT peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 38H to 38J are diagrams of contemplated remodeling steps of the peptide in Figure 38G based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 38K is a diagram depicting an AAT peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 38L to 38N are diagrams of contemplated remodeling steps of the peptide in Figure 38K based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 39, comprising Figures 39A to 39J sets forth exemplary schemes for remodeling glycan structures on glucocerebrosidase. Figure 39A is a diagram depicting the glucocerebrosidase peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 39B to 39F are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 39A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 39G is a diagram depicting the glucocerebrosidase peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 39H to 39K are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 39G based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 40, comprising Figures 40A to 40W, sets forth exemplary schemes for remodeling glycan structures on Tissue-Type Plasminogen Activator (TPA). Figure 40A is a diagram depicting the TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 40B to 40G are diagrams of contemplated remodeling steps of the peptide in Figure 40A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40H is a diagram depicting the TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 40I to 40K are diagrams of contemplated remodeling steps of the peptide in Figure 40H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40L is a diagram depicting a



mutant TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and the formula for the glycans. Figure 40M to 40O are diagrams of contemplated remodeling steps of the peptide in Figure 40L based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40P is a diagram  
5 depicting a mutant TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 40Q to 40S are diagrams of contemplated remodeling steps of the peptide in Figure 40P based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40T is a diagram depicting a mutant TPA peptide indicating the residues which links to glycans contemplated  
10 for remodeling, and formulas for the glycans. Figure 40U to 40W are diagrams of contemplated remodeling steps of the peptide in Figure 40T based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 41, comprising Figures 41A to 41G, sets forth exemplary schemes for remodeling glycan structures on Interleukin-2 (IL-2). Figure 41A is a diagram depicting the  
15 Interleukin-2 peptide indicating the amino acid residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 41B to 41G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 41A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 42, comprising Figures 42A to 42M, sets forth exemplary schemes for  
20 remodeling glycan structures on Factor VIII. Figure 42A are the formulas for the glycans that bind to the N-linked glycosylation sites (A and A') and to the O-linked sites (B) of the Factor VIII peptides. Figure 42B to 42F are diagrams of contemplated remodeling steps of the peptides in Figure 42A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 42G are the formulas for the glycans that bind to  
25 the N-linked glycosylation sites (A and A') and to the O-linked sites (B) of the Factor VIII peptides. Figure 42H to 42M are diagrams of contemplated remodeling steps of the peptides in Figure 42G based on the type of cell the peptide is expressed in and the desired remodeled glycan structures.

Figure 43, comprising Figures 43A to 43L, sets forth exemplary schemes for  
30 remodeling glycan structures on urokinase. Figure 43A is a diagram depicting the urokinase peptide indicating a residue which is linked to a glycan contemplated for remodeling, and an

exemplary glycan formula linked thereto. Figure 43B to 43F are diagrams of contemplated remodeling steps of the peptide in Figure 43A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 43G is a diagram depicting the urokinase peptide indicating a residue which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 43H to 43L are diagrams of contemplated remodeling steps of the peptide in Figure 43G based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 44, comprising Figures 44A to 44J, sets forth exemplary schemes for remodeling glycan structures on human DNase (hDNase). Figure 44A is a diagram depicting the human DNase peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 44B to 44F are diagrams of contemplated remodeling steps of the peptide in Figure 44A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 44G is a diagram depicting the human DNase peptide indicating residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 44H to 44J are diagrams of contemplated remodeling steps of the peptide in Figure 44F based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 45, comprising Figures 45A to 45L, sets forth exemplary schemes for remodeling glycan structures on insulin. Figure 45A is a diagram depicting the insulin peptide mutated to contain an N glycosylation site and an exemplary glycan formula linked thereto. Figure 45B to 45D are diagrams of contemplated remodeling steps of the peptide in Figure 45A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 45E is a diagram depicting insulin-mucin fusion peptides indicating a residue(s) which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 45F to 45H are diagrams of contemplated remodeling steps of the peptide in Figure 45E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 45I is a diagram depicting the insulin-mucin fusion peptides and insulin peptides indicating a residue(s) which is linked to a glycan contemplated for remodeling, and formulas for the glycan. Figure 45J to 45L are diagrams of contemplated remodeling steps of the peptide in Figure 45I based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 46, comprising Figures 46A to 46K, sets forth exemplary schemes for remodeling glycan structures on the M-antigen (preS and S) of the Hepatitis B surface protein (HbsAg). Figure 46A is a diagram depicting the M-antigen peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 46B to 46G are diagrams of contemplated remodeling steps of the peptide in Figure 46A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 46H is a diagram depicting the M-antigen peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 46I to 46K are diagrams of contemplated remodeling steps of the peptide in Figure 46H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 47, comprising Figures 47A to 47K, sets forth exemplary schemes for remodeling glycan structures on human growth hormone, including N, V and variants thereof. Figure 47A is a diagram depicting the human growth hormone peptide indicating a residue which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 47B to 47D are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 47A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 47E is a diagram depicting the three fusion peptides comprising the human growth hormone peptide and part or all of a mucin peptide, and indicating a residue(s) which is linked to a glycan contemplated for remodeling, and exemplary glycan formula(s) linked thereto. Figure 47F to 47K are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 47E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 48, comprising Figures 48A to 48G, sets forth exemplary schemes for remodeling glycan structures on a TNF Receptor-IgG Fc region fusion protein (Enbrel™). Figure 48A is a diagram depicting a TNF Receptor-IgG Fc region fusion peptide which may be mutated to contain additional N-glycosylation sites indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. The TNF receptor peptide is depicted in bold line, and the IgG Fc regions is depicted in regular line. Figure 48B to 48G are diagrams of contemplated remodeling steps of the peptide in Figure 48A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 49, comprising Figures 49A to 49D, sets forth exemplary schemes for remodeling glycan structures on an anti-HER2 monoclonal antibody (Herceptin™). Figure 49A is a diagram depicting an anti-HER2 monoclonal antibody which has been mutated to contain an N-glycosylation site(s) indicating a residue(s) on the antibody heavy chain which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 49B to 49D are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 49A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 50, comprising Figures 50A to 50D, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to Protein F of Respiratory Syncytial Virus (Synagis™). Figure 50A is a diagram depicting a monoclonal antibody to Protein F peptide which is mutated to contain an N-glycosylation site(s) indicating a residue(s) which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 50B to 50D are diagrams of contemplated remodeling steps of the peptide in Figure 50A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 51, comprising Figures 51A to 51D, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to TNF- $\alpha$  (Remicade™). Figure 51A is a diagram depicting a monoclonal antibody to TNF- $\alpha$  which has an N-glycosylation site(s) indicating a residue which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 51B to 51D are diagrams of contemplated remodeling steps of the peptide in Figure 51A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 52, comprising Figures 52A to 52L, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to glycoprotein IIb/IIIa (Reopro™). Figure 52A is a diagram depicting a mutant monoclonal antibody to glycoprotein IIb/IIIa peptides which have been mutated to contain an N-glycosylation site(s) indicating the residue(s) which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 52B to 52D are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 52E is a diagram depicting monoclonal antibody to glycoprotein IIb/IIIa-

mucin fusion peptides indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 52F to 52H are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 52I is a diagram depicting monoclonal antibody to glycoprotein IIb/IIIa- mucin fusion peptides and monoclonal antibody to glycoprotein IIb/IIIa peptides indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 52J to 52L are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 53, comprising Figures 53A to 53G, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to CD20 (Rituxan™). Figure 53A is a diagram depicting monoclonal antibody to CD20 which have been mutated to contain an N-glycosylation site(s) indicating the residue which is linked to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 53B to 53D are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 53A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 53E is a diagram depicting monoclonal antibody to CD20 which has been mutated to contain an N-glycosylation site(s) indicating the residue(s) which is linked to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 53F to 53G are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 53E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 54, comprising Figures 54A to 54O, sets forth exemplary schemes for remodeling glycan structures on anti-thrombin III (AT III). Figure 54A is a diagram depicting the anti-thrombin III peptide indicating the amino acid residues to which an N-linked glycan is linked, and an exemplary glycan formula linked thereto. Figure 54B to 54G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 54A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 54H is a diagram depicting the anti-thrombin III peptide indicating the amino acid residues to which an N-linked glycan is linked, and an exemplary glycan formula linked thereto. Figure 54I to 54K are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 54H based on the type of cell the peptide is expressed in and

the desired remodeled glycan structure. Figure 54L is a diagram depicting the anti-thrombin III peptide indicating the amino acid residues to which an N-linked glycan is linked, and an exemplary glycan formula linked thereto. Figure 54M to 54O are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 54L based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 55, comprising Figures 55A to 55J, sets forth exemplary schemes for remodeling glycan structures on subunits  $\alpha$  and  $\beta$  of human Chorionic Gonadotropin (hCG). Figure 55A is a diagram depicting the hCG $\alpha$  and hCG $\beta$  peptides indicating the residues which bind to N-linked glycans (A) and O-linked glycans (B) contemplated for remodeling, and formulas for the glycans. Figure 55B to 55J are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 56, comprising Figures 56A to 56J, sets forth exemplary schemes for remodeling glycan structures on alpha-galactosidase (Fabrazyme™). Figure 56A is a diagram depicting the alpha-galactosidase A peptide indicating the amino acid residues which bind to N-linked glycans (A) contemplated for remodeling, and formulas for the glycans. Figure 56B to 56J are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 57, comprising Figures 57A to 57J, sets forth exemplary schemes for remodeling glycan structures on alpha-iduronidase (Aldurazyme™). Figure 57A is a diagram depicting the alpha-iduronidase peptide indicating the amino acid residues which bind to N-linked glycans (A) contemplated for remodeling, and formulas for the glycans. Figure 57B to 57J are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 58, comprising Figures 58A and 58B, is an exemplary nucleotide and corresponding amino acid sequence of granulocyte colony stimulating factor (G-CSF) (SEQ ID NOS: 1 and 2, respectively).

Figure 59, comprising Figures 59A through 59D, is an exemplary nucleotide and corresponding amino acid sequence of interferon alpha (IFN-alpha) (Figures 59A and 59 B, SEQ ID NOS: 3 and 4, respectively), and an exemplary nucleotide and corresponding amino

acid sequence of interferon-omega (IFN-omega) (Figures 59C and 59 D, SEQ ID NOS: 74 and 75, respectively).

Figure 60, comprising Figures 60A and 60B, is an exemplary nucleotide and corresponding amino acid sequence of interferon beta (IFN-beta) (SEQ ID NOS: 5 and 6, respectively).

Figure 61, comprising Figures 61A and 61B, is an exemplary nucleotide and corresponding amino acid sequence of Factor VIIa (SEQ ID NOS: 7 and 8, respectively).

Figure 62, comprising Figures 62A and 62B, is an exemplary nucleotide and corresponding amino acid sequence of Factor IX (SEQ ID NOS: 9 and 10, respectively).

Figure 63, comprising Figures 63A through 63D, is an exemplary nucleotide and corresponding amino acid sequence of the alpha and beta chains of follicle stimulating hormone (FSH), respectively (SEQ ID NOS: 11 through 14, respectively).

Figure 64, comprising Figures 64A and 64B, is an exemplary nucleotide and corresponding amino acid sequence of erythropoietin (EPO) (SEQ ID NOS: 15 and 16, respectively).

Figure 65 is an amino acid sequence of mature EPO, i.e. 165 amino acids (SEQ ID NO:73).

Figure 66, comprising Figures 66A and 66B, is an exemplary nucleotide and corresponding amino acid sequence of granulocyte-macrophage colony stimulating factor (GM-CSF) (SEQ ID NOS: 17 and 18, respectively).

Figure 67, comprising Figures 67A and 67B, is an exemplary nucleotide and corresponding amino acid sequence of interferon gamma (IFN-gamma) (SEQ ID NOS: 19 and 20, respectively).

Figure 68, comprising Figures 68A and 68B, is an exemplary nucleotide and corresponding amino acid sequence of  $\alpha$ -1-protease inhibitor (A-1-PI, or  $\alpha$ -antitrypsin) (SEQ ID NOS: 21 and 22, respectively).

Figure 69, comprising Figures 69A-1 to 69A-2, and 69B, is an exemplary nucleotide and corresponding amino acid sequence of glucocerebrosidase (SEQ ID NOS: 23 and 24, respectively).

Figure 70, comprising Figures 70A and 70B, is an exemplary nucleotide and corresponding amino acid sequence of tissue-type plasminogen activator (TPA) (SEQ ID NOS: 25 and 26, respectively).

5 Figure 71, comprising Figures 71A and 71B, is an exemplary nucleotide and corresponding amino acid sequence of Interleukin-2 (IL-2) (SEQ ID NOS: 27 and 28, respectively).

Figure 72, comprising Figures 72A-1 through 72A-4 and Figure 72B-1 through 72B-4, is an exemplary nucleotide and corresponding amino acid sequence of Factor VIII (SEQ ID NOS: 29 and 30, respectively).

10 Figure 73, comprising Figures 73A and 73B, is an exemplary nucleotide and corresponding amino acid sequence of urokinase (SEQ ID NOS: 33 and 34, respectively).

Figure 74, comprising Figures 74A and 74B, is an exemplary nucleotide and corresponding amino acid sequence of human recombinant DNase (hrDNase) (SEQ ID NOS: 39 and 40, respectively).

15 Figure 75, comprising Figures 75A and 75B, is an exemplary nucleotide and corresponding amino acid sequence of an insulin molecule (SEQ ID NOS: 43 and 44, respectively).

Figure 76, comprising Figures 76A and 76B, is an exemplary nucleotide and corresponding amino acid sequence of S-protein from a Hepatitis B virus (HbsAg) (SEQ ID NOS: 45 and 46, respectively).

20 Figure 77, comprising Figures 77A and 77B, is an exemplary nucleotide and corresponding amino acid sequence of human growth hormone (hGH) (SEQ ID NOS: 47 and 48, respectively).

Figure 78, comprising Figures 78A and 78B, are exemplary nucleotide and corresponding amino acid sequences of anti-thrombin III. Figures 78A and 78B, are an exemplary nucleotide and corresponding amino acid sequences of "WT" anti-thrombin III (SEQ ID NOS: 63 and 64, respectively).

Figure 79, comprising Figures 79A to 79D, are exemplary nucleotide and corresponding amino acid sequences of human chorionic gonadotropin (hCG)  $\alpha$  and  $\beta$  subunits. Figures 79A and 79B are an exemplary nucleotide and corresponding amino acid sequence of the  $\alpha$ -subunit of human chorionic gonadotropin (SEQ ID NOS: 69 and 70,



respectively). Figures 79C and 79D are an exemplary nucleotide and corresponding amino acid sequence of the beta subunit of human chorionic gonadotrophin (SEQ ID NOS: 71 and 72, respectively).

Figure 80, comprising Figures 80A and 80B, is an exemplary nucleotide and  
5 corresponding amino acid sequence of  $\alpha$ -iduronidase (SEQ ID NOS: 65 and 66, respectively).

Figure 81, comprising Figures 81A and 81B, is an exemplary nucleotide and corresponding amino acid sequence of  $\alpha$ -galactosidase A (SEQ ID NOS: 67 and 68, respectively).

10 Figure 82, comprising Figures 82A and 82B, is an exemplary nucleotide and corresponding amino acid sequence of the 75 kDa tumor necrosis factor receptor (TNF-R), which comprises a portion of Enbrel™ (tumor necrosis factor receptor (TNF-R)/IgG fusion) (SEQ ID NOS: 31 and 32, respectively).

Figure 83, comprising Figures 83A and 83B, is an exemplary amino acid sequence of  
15 the light and heavy chains, respectively, of Herceptin™ (monoclonal antibody (MAb) to Her-2, human epidermal growth factor receptor) (SEQ ID NOS: 35 and 36, respectively).

Figure 84, comprising Figures 84A and 84B, is an exemplary amino acid sequence the heavy and light chains, respectively, of Synagis™ (MAb to F peptide of Respiratory Syncytial Virus) (SEQ ID NOS: 37 and 38, respectively).

20 Figure 85, comprising Figures 85A and 85B, is an exemplary nucleotide and corresponding amino acid sequence of the non-human variable regions of Remicade™ (MAb to TNF $\alpha$ ) (SEQ ID NOS: 41 and 42, respectively).

Figure 86, comprising Figures 86A and 86B, is an exemplary nucleotide and corresponding amino acid sequence of the Fc portion of human IgG (SEQ ID NOS: 49 and  
25 50, respectively).

Figure 87 is an exemplary amino acid sequence of the mature variable region light chain of an anti-glycoprotein IIb/IIIa murine antibody (SEQ ID NO: 52).

Figure 88 is an exemplary amino acid sequence of the mature variable region heavy chain of an anti-glycoprotein IIb/IIIa murine antibody (SEQ ID NO: 54).

30 Figure 89 is an exemplary amino acid sequence of variable region light chain of a human IgG (SEQ ID NO: 51).

Figure 90 is an exemplary amino acid sequence of variable region heavy chain of a human IgG (SEQ ID NO:53).

Figure 91 is an exemplary amino acid sequence of a light chain of a human IgG (SEQ ID NO:55).

5        Figure 92 is an exemplary amino acid sequence of a heavy chain of a human IgG (SEQ ID NO:56).

Figure 93, comprising Figures 93A and 93B, is an exemplary nucleotide and corresponding amino acid sequence of the mature variable region of the light chain of an anti-CD20 murine antibody (SEQ ID NOS: 59 and 60, respectively).

10       Figure 94, comprising Figures 94A and 94B, is an exemplary nucleotide and corresponding amino acid sequence of the mature variable region of the heavy chain of an anti-CD20 murine antibody (SEQ ID NOS: 61 and 62, respectively).

Figure 95, comprising Figures 95A through 95E, is the nucleotide sequence of the tandem chimeric antibody expression vector TCAE 8 (SEQ ID NO:57).

15       Figure 96, comprising Figures 96A through 96E, is the nucleotide sequence of the tandem chimeric antibody expression vector TCAE 8 containing the light and heavy variable domains of the anti-CD20 murine antibody (SEQ ID NO:58).

Figure 97, comprising Figures 97A to 97C, are graphs depicting 2-AA HPLC analysis of glycans released by PNGaseF from myeloma-expressed Cri-IgG1 antibody. The structure of the glycans is determined by retention time: the G0 glycoform elutes at 30 min., the G1 glycoform elutes at ~ 33 min., the G2 glycoform elutes at about approximately 37 min. and the S1-G1 glycoform elutes at ~ 70 min. Figure 97A depicts the analysis of the DEAE antibody sample. Figure 97B depicts the analysis of the SPA antibody sample. Figure 97C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these  
20       graphs is summarized in Table 14.

Figure 98, comprising Figures 98A to 98C, are graphs depicting the MALDI analysis of glycans released by PNGaseF from myeloma-expressed Cri-IgG1 antibody. The glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 98A depicts the analysis of the DEAE antibody sample. Figure 98B depicts the analysis of the SPA antibody sample.  
30       Figure 98C depicts the analysis of the Fc antibody sample.

Figure 99, comprising Figures 99A to 99D, are graphs depicting the capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain M3N2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 99A.

5 Figure 99B depicts the analysis of the DEAE antibody sample. Figure 99C depicts the analysis of the SPA antibody sample. Figure 99D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 15.

Figure 100, comprising Figures 100A to 100D, are graphs depicting the capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G0 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 100A. Figure 100B depicts the analysis of the DEAE antibody sample. Figure 100C depicts the analysis of the SPA antibody sample. Figure 100D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 16.

15 Figure 101, comprising Figures 101A to 101C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G0 glycoforms. The released glycans were labeled with 2AA and separated by HPLC on a NH2P-50 4D amino column. Figure 101A depicts the analysis of the DEAE antibody sample. Figure 101B depicts the analysis of the SPA antibody sample. Figure 101C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 16

Figure 102, comprising Figures 102A to 102C, are graphs depicting the MALDI analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G0 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 102A depicts the analysis of the DEAE antibody sample. Figure 102B depicts the analysis of the SPA antibody sample. Figure 102C depicts the analysis of the Fc antibody sample.

Figure 103, comprising Figures 103A to 103D, are graphs depicting the capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 103A. Figure 103B

depicts the analysis of the DEAE antibody sample. Figure 103C depicts the analysis of the SPA antibody sample. Figure 103D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 17.

Figure 104, comprising Figures 104A to 104C, are graphs depicting the 2-AA HPLC analysis of glycans released from remodeled Cri-IgG1 antibodies that have been glycoremodeled to contain G2 glycoforms. The released glycans were labeled with 2AA and then separated by HPLC on a NH2P-50 4D amino column. Figure 104A depicts the analysis of the DEAE antibody sample. Figure 104B depicts the analysis of the SPA antibody sample. Figure 104C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 17.

Figure 105, comprising Figures 105A to 105C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 105A depicts the analysis of the DEAE antibody sample. Figure 105B depicts the analysis of the SPA antibody sample. Figure 105C depicts the analysis of the Fc antibody sample.

Figure 106, comprising Figures 106A to 106D, are graphs depicting capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I treatment of M3N2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 106A. Figure 106B depicts the analysis of the DEAE antibody sample. Figure 106C depicts the analysis of the SPA antibody sample. Figure 106D depicts the analysis of the Fc antibody sample.

Figure 107, comprising Figures 107A to 107C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been remodeled by GnT-I treatment of M3N2 glycoforms. The released glycans were labeled with 2-AA and separated by HPLC on a NH2P-50 4D amino column. Figure 107A depicts the analysis of the DEAE antibody sample. Figure 107B depicts the analysis of the SPA antibody sample. Figure 107C depicts the analysis of the Fc antibody sample.

Figure 108, comprising Figures 108A to 108C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I

treatment of M3N2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 108A depicts the analysis of the DEAE antibody sample. Figure 108B depicts the analysis of the SPA antibody sample. Figure 108C depicts the analysis of the Fc antibody sample.

5        Figure 109, comprising Figures 109A to 109D, are graphs depicting capillary electrophoresis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I, II and III treatment of M3N2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 109A. Figure 109B depicts the analysis of the DEAE antibody sample. Figure 109C depicts the  
10      analysis of the SPA antibody sample. Figure 109D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 18.

      Figure 110, comprising Figures 110A to 110C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I, II and III treatment of M3N2 glycoforms. The released glycans were labeled with  
15      2AA and then separated by HPLC on a NH2P-50 4D amino column. Figure 110A depicts the analysis of the DEAE antibody sample. Figure 110B depicts the analysis of the SPA antibody sample. Figure 110C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 18.

      Figure 111, comprising Figures 111A to 111C, are graphs depicting MALDI analysis  
20      of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by galactosyltransferase treatment of NGA2F glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 111A depicts the analysis of the DEAE antibody sample. Figure 111B depicts the analysis of the SPA antibody sample. Figure 111C depicts the analysis of the Fc antibody sample.

25      Figure 112, comprising 112A to 112D, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies containing NGA2F isoforms before GalT1 treatment (Figures 112A and 112C) and after GalT1 treatment (Figures 112B and 112D). Figures 112A and 112B depict the analysis of the DEAE sample of antibodies. Figures 112C and 112D depict the analysis of the Fc sample of antibodies. The released glycans were  
30      labeled with 2AA and separated by HPLC on a NH2P-50 4D amino column.

Figure 113, comprising 113A to 113C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by ST3Gal3 treatment of G2 glycoforms. The released glycans are labeled with 2-AA and then separated by HPLC on a NH2P-50 4D amino column. Figure 113A depicts the analysis of the DEAE antibody sample. Figure 113B depicts the analysis of the SPA antibody sample. Figure 113C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 19.

Figure 114, comprising Figures 114A to 114C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST3Gal3 treatment of G2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 114A depicts the analysis of the DEAE antibody sample. Figure 114B depicts the analysis of the SPA antibody sample. Figure 114C depicts the analysis of the Fc antibody sample.

Figure 115, comprising Figures 115A to 115D, are graphs depicting capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST6Gal1 treatment of G2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 115A. Figure 115B depicts the analysis of the DEAE antibody sample. Figure 115C depicts the analysis of the SPA antibody sample. Figure 115D depicts the analysis of the Fc antibody sample.

Figure 116, comprising Figures 116A to 116C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST6Gal1 treatment of G2 glycoforms. The released glycans were labeled with 2-AA and separated by HPLC on a NH2P-50 4D amino column. Figure 116A depicts the analysis of the DEAE antibody sample. Figure 116B depicts the analysis of the SPA antibody sample. Figure 116C depicts the analysis of the Fc antibody sample.

Figure 117, comprising Figures 117A to 117C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST6Gal1 treatment of G2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 117A depicts the analysis of the DEAE antibody sample.

Figure 117B depicts the analysis of the SPA antibody sample. Figure 117C depicts the analysis of the Fc antibody sample.

Figure 118, comprising Figures 118A to 118E, depicts images of SDS-PAGE analysis of the glycoremodeled of Cri-IgG1 antibodies with different glycoforms under non-reducing conditions. Bovine serum albumin (BSA) was run under reducing conditions as a quantitative standard. Protein molecular weight standards are displayed and their size is indicated in kDa. Figure 118A depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain G0 and G2 glycoforms. Figure 118B depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain NGA2F (bisecting) and GnT-I-M3N2 (GnT1) glycoforms. Figure 118C depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain S2G2 (ST6Gal1) glycoforms. Figure 118D depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain M3N2 glycoforms, and BSA. Figure 118E depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain Gal-NGA2F (Gal-bisecting) glycoforms, and BSA.

Figure 119 is an image of an acrylamide gel depicting the results of FACE analysis of the pre- and post-sialylation of TP10. The BiNA<sub>0</sub> species has no sialic acid residues. The BiNA<sub>1</sub> species has one sialic acid residue. The BiNA<sub>2</sub> species has two sialic acid residues. Bi = biantennary; NA = neuraminic acid.

Figure 120 is a graph depicting the plasma concentration in µg/ml over time of pre- and post-sialylation TP10 injected into rats.

Figure 121 is a graph depicting the area under the plasma concentration-time curve (AUC) in µg/hr/ml for pre- and post sialylated TP10.

Figure 122 is an image of an acrylamide gel depicting the results of FACE glycan analysis of the pre- and post-fucosylation of TP10 and FACE glycan analysis of CHO cell produced TP-20. The BiNA<sub>2</sub>F<sub>2</sub> species has two neuraminic acid (NA) residues and two fucose residues (F).

Figure 123 is a graph depicting the *in vitro* binding of TP20 (sCR1sLe<sup>X</sup>) glycosylated *in vitro* (diamonds) and *in vivo* in Lec11 CHO cells (squares).

Figure 124 is a graph depicting the analysis by 2-AA HPLC of glycoforms from the GlcNAc-ylation of EPO.

Figure 125, comprising Figures 125A and 125B, are graphs depicting the 2-AA HPLC analysis of two lots of EPO to which N-acetylglucosamine was been added. Figure 125A depicts the analysis of lot A, and Figure 125B depicts the analysis of lot B.

Figure 126 is a graph depicting the 2-AA HPLC analysis of the products the reaction  
5 introducing a third glycan branch to EPO with GnT-V.

Figure 127 is a graph depicting a MALDI-TOF spectrum of the glycans of the EPO preparation after treatment with GnT-I, GnT-II, GnT-III, GnT-V and GalT1, with appropriate donor groups.

Figure 128 is a graph depicting a MALDI spectrum the glycans of native EPO.

10 Figure 129 is an image of an SDS-PAGE gel of the products of the PEGylation reactions using CMP-SA-PEG (1 kDa), and CMP-SA-PEG (10 kDa).

Figure 130 is a graph depicting the results of the *in vitro* bioassay of PEGylated EPO. Diamonds represent the data from sialylated EPO having no PEG molecules. Squares represent the data obtained using EPO with PEG (1 kDa). Triangles represent the data  
15 obtained using EPO with PEG (10 kDa).

Figure 131 is a diagram of CHO-expressed EPO. The EPO polypeptide is 165 amino acids in length, with a molecular weight of 18 kDa without glycosylation. The glycosylated forms of EPO produced in CHO cells have a molecular weight of about 33 kDa to 39 kDa. The shapes which represent the sugars in the glycan chains are identified in the box at the  
20 lower edge of the drawing.

Figure 132 is a diagram of insect cell expressed EPO. The shapes that represent the sugars in the glycan chains are identified in the box at the lower edge of FIG. 131.

Figure 133 is a bar graph depicting the molecular weights of the EPO peptides expressed in insect cells which were remodeled to form complete mono-, bi- and tri-  
25 antennary glycans, with optional glycoPEGylation with 1 kDa, 10 kDa or 20 kDa PEG. Epoetin™ is EPO expressed in mammalian cells without further glycan modification or PEGylation. NESP (Aranesp™, Amgen, Thousand Oaks, CA) is a form of EPO having 5 N-linked glycan sites that is also expressed in mammalian cells without further glycan modification or PEGylation.

30 Figure 134, comprising Figures 134A and 134B, depicts one scheme for the remodeling and glycoPEGylation of insect cell expressed EPO. Figure 134A depicts the



remodeling and glycoPEGylation steps that remodel the insect expressed glycan to a mono-antennary glycoPEGylated glycan. Figure 134B depicts the remodeled EPO polypeptide having a completed glycoPEGylated mono-antennary glycan at each N-linked glycan site of the polypeptide. The shapes that represent the sugars in the glycan chains are identified in the box at the lower edge of FIG. 131, except that the triangle represents sialic acid.

Figure 135 is a graph depicting the *in vitro* bioactivities of EPO-SA and EPO-SA-PEG constructs. The *in vitro* assay measured the proliferation of TF-1 erythroleukemia cells which were maintained for 48 hr in RBMI + FBS 10% + GM-CSF (12 ng/ml) after the EPO construct was added at 10.0, 5.0, 2.0, 1.0, 0.5, and 0 µg/ml. Tri-SA refers to EPO constructs where the glycans are tri-antennary and have SA, Tri-SA 1K PEG refers to EPO constructs where the glycans are tri-antennary and have Gal and are then glycoPEGylated with SA-PEG 1 kDa. Di-SA 10K PEG refers to EPO constructs where the glycans are bi-antennary and have Gal and are then glycoPEGylated with SA-PEG 10 kDa. Di-SA 1K PEG refers to EPO constructs where the glycans are bi-antennary and have Gal and are then glycoPEGylated with SA-PEG 1 kDa. Di-SA refers to EPO constructs where the glycans are bi-antennary and are built out to SA. Epogen™ is EPO expressed in CHO cells with no further glycan modification.

Figure 136 is a graph depicting the pharmacokinetics of the EPO constructs in rat. Rats were bolus injected with [<sup>125</sup>I]-labeled glycoPEGylated and non-glycoPEGylated EPO. The graph shows the concentration of the radio-labeled EPO in the bloodstream of the rat at 0 to about 72 minutes after injection. "Biant-10K" refers to EPO with biantennary glycan structures with terminal 10 kDa PEG moieties. "Mono-20K" refers to EPO with monoantennary glycan structures with terminal 20 kDa PEG moieties. NESP refers to the commercially available Aranesp. "Biant-1K" refers to EPO with biantennary glycan structures with terminal 1 kDa PEG moieties. "Biant-SA" refers to EPO with biantennary glycan structures with terminal 1 kDa moieties. The concentration of the EPO constructs in the bloodstream at 72 hr. is as follows: Biant-10K, 5.1 cpm/ml; Mono-20K, 3.2 cpm/ml; NESP, 1 cpm/ml; and Biant-1K, 0.2 cpm/ml; Biant-SA, 0.1 cpm/ml. The relative area under the curve of the EPO constructs is as follows: Biant-10K, 2.9; Mono-20K, 2.1; NESP, 1; Biant-1K, 0.5; and Biant-SA, 0.2.

Figure 137 is a bar graph depicting the ability of the EPO constructs to stimulate reticulocytosis *in vivo*. Each treatment group is composed of eight mice. Mice were given a single subcutaneous injection of 10 µg protein / kg body weight. The percent reticulocytosis was measured at 96 hr. Tri-antennary-SA2,3(6) construct has the SA molecule bonded in a 2,3 or 2,6 linkage (see, Example 18 herein for preparation) wherein the glycan on EPO is tri-antennary N-glycans with SA-PEG 10 K is attached thereon. Similarly, bi-antennary-10K PEG is EPO having a bi-antennary N-glycan with SA-PEG at 10 K PEG attached thereon.

Figure 138 is a bar graph depicting the ability of EPO constructs to increase the hematocrit of the blood of mice *in vivo*. CD-1 female mice were injected i.p. with 2.5 µg protein/kg body weight. The hematocrit of the mice was measured on day 15 after the EPO injection. Bi-1k refers to EPO constructs where the glycans are bi-antennary and are built out to the Gal and then glycoPEGylated with SA-PEG 1 kDa. Mono-20k refers to EPO constructs where the glycans are mono-antennary and are built out to the Gal and then glycoPEGylated with SA-PEG 20 kDa.

Figure 139, comprising Figures 139A and 139B, depicts the analysis of glycans enzymatically released from EPO expressed in insect cells (Protein Sciences, Lot # 060302). Figure 139A depicts the HPLC analysis of the released glycans. Figure 139B depicts the MALDI analysis of the released glycans. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

Figure 140 depicts the MALDI analysis of glycans released from EPO after the GnT-I/GalT-1 reaction. The structures of the glycans have been determined by comparison of the peak spectrum with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose, stars represent galactose.

Figure 141 depicts the SDS-PAGE analysis of EPO after the GnT-I/GalT-1 reaction, Superdex 75 purification, ST3Gal3 reaction with SA-PEG (10 kDa) and SA-PEG (20 kDa).

Figure 142 depicts the results of the TF-1 cell *in vitro* bioassay of PEGylated mono-antennary EPO.

Figure 143, comprising Figures 143A and 143B, depicts the analysis of glycan released from EPO after the GnT-I/GnT-II reaction. Figure 143A depicts the HPLC analysis of the released glycans, where peak 3 represents the bi-antennary GlcNAc glycan. Figure

143B depicts the MALDI analysis of the released glycans. The structures of the glycans have been determined by comparison of the peak spectrum with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

5        Figure 144, comprising Figures 144A and 144B, depict the HPLC analysis of glycans released from EPO after the GalT-1 reaction. Figure 144A depicts the glycans released after the small scale GalT-1 reaction. Figure 144B depicts the glycans released after the large scale GalT-1 reaction. In both figures, Peak 1 is the bi-antennary glycan with terminal galactose moieties and Peak 2 is the bi-antennary glycan without terminal galactose moieties.

10       Figure 145 depicts the Superdex 75 chromatography separation of EPO species after the GalT-1 reaction. Peak 2 contains EPO with bi-antennary glycans with terminal galactose moieties.

      Figure 146 depicts the SDS-PAGE analysis of each of the products of the glycoremodeling process to make bi-antennary glycans with terminal galactose moieties.

15       Figure 147 depicts the SDS-PAGE analysis of EPO after ST3Gal3 sialylation or PEGylation with SA-PEG (1 kDa) or SA-PEG (10 kDa).

      Figure 148 depicts the HPLC analysis of glycans released from EPO after the GnT-I/GnT-II reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

20       Figure 149 depicts the HPLC analysis of glycans released from EPO after the GnT-V reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

25       Figure 150 depicts the HPLC analysis of glycans released from EPO after the GalT-1 reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose, open circles represent galactose and triangles represent sialic acid.

Figure 151 depicts the HPLC analysis of glycans released from EPO after the ST3Gal3 reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose,  
5 open circles represent galactose and triangles represent sialic acid.

Figure 152 depicts the HPLC analysis of glycans released from EPO after the ST6Gal1 reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks.

10 Figure 153 depicts the results of the TF-1 cells *in vitro* bioassay of EPO with bi-antennary and triantennary glycans. "Di-SA" refers to EPO with bi-antennary glycans that terminate in sialic acid. "Di-SA 10K PEG" refers to EPO with bi-antennary glycans that terminate in sialic acid derivatized with PEG (10 kDa). "Di-SA 1K PEG" refers to EPO with bi-antennary glycans that terminate in sialic acid derivatized with PEG (1 kDa). "Tri-SA ST6  
15 + ST3" refers to EPO with tri-antennary glycans terminating in 2,6-SA capped with 2,3-SA. "Tri-SA ST3" refers to EPO with tri-antennary glycans terminating in 2,3-SA.

Figure 154 is an image of an IEF gel depicting the pI of the products of the desialylation procedure. Lanes 1 and 5 are IEF standards. Lane 2 is Factor IX protein. Lane 3 is rFactor IX protein. Lane 4 is the desialylation reaction of rFactor IX protein at 20 hr.

20 Figure 155 is an image of an SDS-PAGE gel depicting the molecular weight of Factor IX conjugated with either SA-PEG (1 kDa) or SA-PEG (10 kDa) after reaction with CMP-SA-PEG. Lanes 1 and 6 are SeeBlue +2 molecular weight standards. Lane 2 is rF-IX. Lane 3 is desialylated rF-IX. Lane 4 is rFactor IX conjugated to SA-PEG (1 kDa). Lane 5 is rFactor IX conjugated to SA-PEG (10 kDa).

25 Figure 156 is an image of an SDS-PAGE gel depicting the reaction products of direct-sialylation of Factor-IX and sialic acid capping of Factor-IX-SA-PEG. Lane 1 is protein standards, lane 2 is blank; lane 3 is rFactor-IX; lane 4 is SA capped rFactor-IX-SA-PEG (10 kDa); lane 5 is rFactor-IX-SA-PEG (10 kDa); lane 6 is ST3Gal1; lane 7 is ST3Gal3; lanes 8, 9, 10 are rFactor-IX-SA-PEG(10 kDa) with no prior sialidase treatment.

30 Figure 157 is an image of an isoelectric focusing gel (pH 3-7) of asialo-Factor VIIa. Lane 1 is rFactor VIIa; lanes 2-5 are asialo-Factor VIIa.

Figure 158 is a graph of a MALDI spectra of Factor VIIa.

Figure 159 is a graph of a MALDI spectra of Factor VIIa-PEG (1 kDa).

Figure 160 is a graph depicting a MALDI spectra of Factor VIIa-PEG (10 kDa).

Figure 161 is an image of an SDS-PAGE gel of PEGylated Factor VIIa. Lane 1 is  
5 asialo-Factor VIIa. Lane 2 is the product of the reaction of asialo-Factor VIIa and CMP-SA-  
PEG(1 kDa) with ST3Gal3 after 48 hr. Lane 3 is the product of the reaction of asialo-Factor  
VIIa and CMP-SA-PEG (1 kDa) with ST3Gal3 after 48 hr. Lane 4 is the product of the  
reaction of asialo-Factor VIIa and CMP-SA-PEG (10 kDa) with ST3Gal3 at 96 hr.

Figure 162 is an image of an isoelectric focusing (IEF) gel depicting the products of  
10 the desialylation reaction of human pituitary FSH. Lanes 1 and 4 are isoelectric focusing  
(IEF) standards. Lane 2 is native FSH. Lane 3 is desialylated FSH.

Figure 163 is an image of an SDS-PAGE gel of the products of the reactions to make  
PEG-sialylation of rFSH. Lanes 1 and 8 are SeeBlue+2 molecular weight standards. Lane 2  
is 15 µg of native FSH. Lane 3 is 15 µg of asialo-FSH (AS-FSH). Lane 4 is 15 µg of the  
15 products of the reaction of AS-FSH with CMP-SA. Lane 5 is 15 µg of the products of the  
reaction of AS-FSH with CMP-SA-PEG (1 kDa). Lane 6 is 15 µg of the products of the  
reaction of AS-FSH with CMP-SA-PEG (5 kDa). Lane 7 is 15 µg of the products of the  
reaction of AS-FSH with CMP-SA-PEG (10 kDa).

Figure 164 is an image of an isoelectric focusing gel of the products of the reactions  
20 to make PEG-sialylation of FSH. Lanes 1 and 8 are IEF standards. Lane 2 is 15 µg of native  
FSH. Lane 3 is 15 µg of asialo-FSH (AS-FSH). Lane 4 is 15 µg of the products of the  
reaction of AS-FSH with CMP-SA. Lane 5 is 15 µg of the products of the reaction of AS-  
FSH with CMP-SA-PEG (1 kDa). Lane 6 is 15 µg of the products of the reaction of AS-FSH  
with CMP-SA-PEG (5 kDa). Lane 7 is 15 µg of the products of the reaction of AS-FSH with  
25 CMP-SA-PEG (10 kDa).

Figure 165 is an image of an SDS-PAGE gel of native non-recombinant FSH  
produced in human pituitary cells. Lanes 1, 2 and 5 are SeeBlue™+2 molecular weight  
standards. Lanes 3 and 4 are native FSH at 5 µg and 25 µg, respectively.

Figure 166 is an image of an isoelectric focusing gel (pH 3-7) depicting the products  
30 of the asialylation reaction of rFSH. Lanes 1 and 4 are IEF standards. Lane 2 is native rFSH.  
Lane 3 is asialo-rFSH.

Figure 167 is an image of an SDS-PAGE gel depicting the results of the PEG-sialylation of asialo-rFSH. Lane 1 is native rFSH. Lane 2 is asialo-FSH. Lane 3 is the products of the reaction of asialo-FSH and CMP-SA. Lanes 4-7 are the products of the reaction between asialoFSH and 0.5 mM CMP-SA-PEG (10 kDa) at 2 hr, 5 hr, 24 hr, and 48 hr, respectively. Lane 8 is the products of the reaction between asialo-FSH and 1.0 mM CMP-SA-PEG (10 kDa) at 48 hr. Lane 9 is the products of the reaction between asialo-FSH and 1.0 mM CMP-SA-PEG (1 kDa) at 48 hr.

Figure 168 is an image of an isoelectric focusing gel showing the products of PEG-sialylation of asialo-rFSH with a CMP-SA-PEG (1 kDa). Lane 1 is native rFSH. Lane 2 is asialo-rFSH. Lane 3 is the products of the reaction of asialo-rFSH and CMP-SA at 24 hr. Lanes 4-7 are the products of the reaction of asialo-rFSH and 0.5 mM CMP-SA-PEG (1 kDa) at 2 hr, 5 hr, 24 hr, and 48 hr, respectively. Lane 8 is blank. Lanes 9 and 10 are the products of the reaction at 48 hr of asialo-rFSH and CMP-SA-PEG (10 kDa) at 0.5 mM and 1.0 mM, respectively.

Figure 169 is graph of the pharmacokinetics of rFSH and rFSH-SA-PEG (1 kDa and 10 kDa). This graph illustrates the relationship between the time a rFSH compound is in the blood stream of the rat, and the mean concentration of the rFSH compound in the blood for glycoPEGylated rFSH as compared to non-PEGylated rFSH.

Figure 170 is a graph of the results of the FSH bioassay using Sertoli cells. This graph illustrates the relationship between the FSH concentration in the Sertoli cell incubation medium and the amount of 17- $\beta$  estradiol released from the Sertoli cells.

Figure 171 is a graph depicting the results of the Steelman-Pohley bioassay of glycoPEGylated and non-glycoPEGylated FSH. Rats were subcutaneously injected with human chorionic gonadotropin and varying amounts of FSH for three days, and the average ovarian weight of the treatment group determined on day 4. rFSH-SA-PEG refers to recombinant FSH that has been glycoPEGylated with PEG (1 kDa). rFSH refers to non-glycoPEGylated FSH. Each treatment group contains 10 rats.

Figure 172, comprising Figures 172A and 172B, depicts the chromatogram of INF- $\beta$  elution from a Superdex-75 column. Figure 172A depicts the entire chromatogram. Figure 172B depicts the boxed area of Figure 172A containing peaks 4 and 5 in greater detail.

Figure 173, comprising Figures 173A and 173B, depict MALDI analysis of glycans enzymatically released from INF- $\beta$ . Figure 173A depicts the MALDI analysis glycans released from native INF- $\beta$ . Figure 173B depicts the MALDI analysis of glycans released from desialylated INF- $\beta$ . The structures of the glycans have been determined by comparison of the peak spectrum with that of standard glycans. The glycan structures are depicted beside the peaks. Squares represent GlcNAc, triangles represent fucose, circles represent mannose, diamonds represent galactose and stars represent sialic acid.

Figure 174 depicts the lectin blot analysis of the sialylation of the desialylated INF- $\beta$ . The blot on the right side is detected with *Maackia amurensis* agglutinin (MAA) labeled with digoxigenin (DIG) (Roche Applied Science, Indianapolis, IL) to detect  $\alpha$ 2,3-sialylation. The blot on the left is detected with *Erthrina cristagalli* lectin (ECL) labeled with biotin (Vector Laboratories, Burlingame, CA) to detect exposed galactose residues.

Figure 175 depicts the SDS-PAGE analysis of the products of the PEG (10 kDa) PEGylation reaction of INF- $\beta$ . "-PEG" refers to INF- $\beta$  before the PEGylation reaction. "+PEG" refers to INF- $\beta$  after the PEGylation reaction.

Figure 176 depicts the SDS-PAGE analysis of the products of the PEG (20 kDa) PEGylation reaction of INF- $\beta$ . "Unmodified" refers to INF- $\beta$  before the PEGylation reaction. "Pegylated" refers to INF- $\beta$  after the PEGylation reaction.

Figure 177 depicts the chromatogram of PEG (10 kDa) PEGylated INF- $\beta$  elution from a Superdex-200 column.

Figure 178 depicts the results of a bioassay of peak fractions of PEG (10 kDa) PEGylated INF- $\beta$  shown in the chromatogram depicted Figure INF-PEG 6.

Figure 179 depicts the chromatogram of PEG (20 kDa) PEGylated INF- $\beta$  elution from a Superdex-200 column.

Figure 180, comprising Figures 180A and 180B, is two graphs depicting the MALDI-TOF spectrum of RNaseB (Figure 180A) and the HPLC profile of the oligosaccharides cleaved from RNaseB by N-Glycanase (Figure 180B). The majority of N-glycosylation sites of the peptide are modified with high mannose oligosaccharides consisting of 5 to 9 mannose residues.

Figure 181 is a scheme depicting the conversion of high mannose N-Glycans to hybrid N-Glycans. Enzyme 1 is  $\alpha$ 1,2-mannosidase, from *Trichodoma reesei* or *Aspergillus saitoi*. Enzyme 2 is GnT-I ( $\beta$ -1,2-N-acetyl glucosaminyl transferase I). Enzyme 3 is GalT-I ( $\beta$ 1,4-galactosyltransferase 1). Enzyme 4 is  $\alpha$ 2,3-sialyltransferase or  $\alpha$ 2,6-sialyltransferase.

5 Figure 182, comprising Figures 182A and 182B, is two graphs depicting the MALDI-TOF spectrum of RNaseB treated with a recombinant *T. reesei*  $\alpha$ 1,2-mannosidase (Figure 182A) and the HPLC profile of the oligosaccharides cleaved by N-Glycanase from the modified RNaseB (Figure 182B).

10 Figure 183 is a graph depicting the MALDI-TOF spectrum of RNaseB treated with a commercially available  $\alpha$ 1,2-mannosidase purified from *A. saitoi* (Glyko & CalBioChem).

Figure 184 is a graph depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 182 with a recombinant GnT-I (GlcNAc transferase-I).

Figure 185 is a graph depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 184 with a recombinant GalT 1 (galactosyltransferase 1).

15 Figure 186 is a graph depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 185 with a recombinant ST3Gal III ( $\alpha$ 2,3-sialyltransferase III) using CMP-SA as the donor for the transferase.

20 Figure 187, comprising Figures 187A and 187B, are graphs depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 185 with a recombinant ST3Gal III ( $\alpha$ 2,3-sialyltransferase III) using CMP-SA-PEG (10 kDa) as the donor for the transferase.

Figure 188 is a series of schemes depicting the conversion of high mannose N-glycans to complex N-glycans. Enzyme 1 is  $\alpha$ 1,2-mannosidase from *Trichoderma reesei* or *Aspergillus saitoi*. Enzyme 2 is GnT-I. Enzyme 3 is GalT 1. Enzyme 4 is  $\alpha$ 2,3-sialyltransferase or  $\alpha$ 2,6-sialyltransferase. Enzyme 5 is  $\alpha$ -mannosidase II. Enzyme 6 is  $\alpha$ -mannosidase. Enzyme 7 is GnT-II. Enzyme 8 is  $\alpha$ 1,6-mannosidase. Enzyme 9 is  $\alpha$ 1,3-mannosidase.

30 Figure 189 is a diagram of the linkage catalyzed by N-acetylglucosaminyltransferase I to VI (GnT I-VI). R = GlcNAc $\beta$ 1,4GlcNAc-Asn-X.



Figure 190 is an image of an SDS-PAGE gel: standard (Lane 1); native transferrin (Lane 2); asialotransferrin (Lane 3); asialotransferrin and CMP-SA (Lane 4); Lanes 5 and 6, asialotransferrin and CMP-SA-PEG (1 kDa) at 0.5 mM and 5 mM, respectively; Lanes 7 and 8, asialotransferrin and CMP-SA-PEG (5 kDa) at 0.5 mM and 5 mM, respectively; Lanes 9 and 10, asialotransferrin and CMP-SA-PEG (10 kDa) at 0.5 mM and 5 mM, respectively.

Figure 191 is an image of an IEF gel: native transferrin (Lane 1); asialotransferrin (Lane 2); asialotransferrin and CMP-SA, 24 hr (Lane 3); asialotransferrin and CMP-SA, 96 hr (Lane 4) Lanes 5 and 6, asialotransferrin and CMP-SA-PEG (1 kDa) at 24 hr and 96 hr, respectively; Lanes 7 and 8, asialotransferrin and CMP-SA-PEG (5 kDa) at 24 hr and 96 hr, respectively; Lanes 9 and 10, asialotransferrin and CMP-SA-PEG (10 kDa) at 24 hr and 96 hr, respectively.

Figure 192 is a graph depicting the effects of EPO-SA-PEG (10 kDa), EPO-SA-PEG (20 kDa), and NESP on hemoglobin response in Sprague Dawley rats. Rats were dosed (indicated by arrows) by subcutaneous injection of 50 µg protein per Kg body weight. Values plotted are mean (n=5/group) increment in total blood hemoglobin baseline corrected against values obtained for control animals injected with vehicle alone.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention includes methods and compositions for the cell free *in vitro* addition and/or deletion of sugars to or from a peptide molecule in such a manner as to provide a glycopeptide molecule having a specific customized or desired glycosylation pattern, wherein the glycopeptide is produced at an industrial scale. In a preferred embodiment of the invention, the glycopeptide so produced has attached thereto a modified sugar that has been added to the peptide via an enzymatic reaction. A key feature of the invention is to take a peptide produced by any cell type and generate a core glycan structure on the peptide, following which the glycan structure is then remodeled *in vitro* to generate a glycopeptide having a glycosylation pattern suitable for therapeutic use in a mammal. More specifically, it is possible according to the present invention, to prepare a glycopeptide molecule having a modified sugar molecule or other compound conjugated thereto, such that

the conjugated molecule confers a beneficial property on the peptide. According to the present invention, the conjugate molecule is added to the peptide enzymatically because enzyme-based addition of conjugate molecules to peptides has the advantage of regioselectivity and stereoselectivity. The glycoconjugate may be added to the glycan on a peptide before or after glycosylation has been completed. In other words, the order of glycosylation with respect to glycoconjugation may be varied as described elsewhere herein. It is therefore possible, using the methods and compositions provided herein, to remodel a peptide to confer upon the peptide a desired glycan structure preferably having a modified sugar attached thereto. It is also possible, using the methods and compositions of the invention to generate peptide molecules having desired and or modified glycan structures at an industrial scale, thereby, for the first time, providing the art with a practical solution for the efficient production of improved therapeutic peptides.

#### Definitions

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (e.g., Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), which are provided throughout this document. The nomenclature used herein and the laboratory procedures used in analytical chemistry and organic syntheses described below are those well known and commonly employed in the art. Standard techniques or modifications thereof, are used for chemical syntheses and chemical analyses.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen. Antibodies can be intact

immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)<sub>2</sub>, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property by which it is characterized. A functional enzyme, for example, is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

As used herein, the structure " $\left\{ \begin{array}{c} \text{ } \end{array} \right\} \text{---AA}$ ", is the point of connection between an amino acid or an amino acid sidechain in the peptide chain and the glycan structure.

"N-linked" oligosaccharides are those oligosaccharides that are linked to a peptide backbone through asparagine, by way of an asparagine-N-acetylglucosamine linkage. N-linked oligosaccharides are also called "N-glycans." All N-linked oligosaccharides have a common pentasaccharide core of Man<sub>3</sub>GlcNAc<sub>2</sub>. They differ in the presence of, and in the number of branches (also called antennae) of peripheral sugars such as N-acetylglucosamine, galactose, N-acetylgalactosamine, fucose and sialic acid. Optionally, this structure may also contain a core fucose molecule and/or a xylose molecule.

An "elemental trimannosyl core structure" refers to a glycan moiety comprising solely a trimannosyl core structure, with no additional sugars attached thereto. When the term

"elemental" is not included in the description of the "trimannosyl core structure," then the glycan comprises the trimannosyl core structure with additional sugars attached thereto. Optionally, this structure may also contain a core fucose molecule and/or a xylose molecule.

5 The term "elemental trimannosyl core glycopeptide" is used herein to refer to a glycopeptide having glycan structures comprised primarily of an elemental trimannosyl core structure. Optionally, this structure may also contain a core fucose molecule and/or a xylose molecule.

10 "O-linked" oligosaccharides are those oligosaccharides that are linked to a peptide backbone through threonine, serine, hydroxyproline, tyrosine, or other hydroxy-containing amino acids.

All oligosaccharides described herein are described with the name or abbreviation for the non-reducing saccharide (i.e., Gal), followed by the configuration of the glycosidic bond ( $\alpha$  or  $\beta$ ), the ring bond (1 or 2), the ring position of the reducing saccharide involved in the bond (2, 3, 4, 6 or 8), and then the name or abbreviation of the reducing saccharide (i.e.,  
15 GlcNAc). Each saccharide is preferably a pyranose. For a review of standard glycobiology nomenclature see, *Essentials of Glycobiology* Varki et al. eds., 1999, CSHL Press.

The term "sialic acid" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often  
20 abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.* (1986) *J. Biol. Chem.* 261: 11550-11557; Kanamori *et al.*, *J. Biol. Chem.* 265: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C<sub>1</sub>-C<sub>6</sub> acyl-Neu5Ac like  
25 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, see, e.g., Varki, *Glycobiology* 2: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

A peptide having "desired glycosylation", as used herein, is a peptide that comprises one or more oligosaccharide molecules which are required for efficient biological activity of the peptide.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate.

The "area under the curve" or "AUC", as used herein in the context of administering a peptide drug to a patient, is defined as total area under the curve that describes the concentration of drug in systemic circulation in the patient as a function of time from zero to infinity.

The term "half-life" or " $t_{1/2}$ ", as used herein in the context of administering a peptide drug to a patient, is defined as the time required for plasma concentration of a drug in a patient to be reduced by one half. There may be more than one half-life associated with the peptide drug depending on multiple clearance mechanisms, redistribution, and other mechanisms well known in the art. Usually, alpha and beta half-lives are defined such that the alpha phase is associated with redistribution, and the beta phase is associated with clearance. However, with protein drugs that are, for the most part, confined to the bloodstream, there can be at least two clearance half-lives. For some glycosylated peptides, rapid beta phase clearance may be mediated via receptors on macrophages, or endothelial cells that recognize terminal galactose, N-acetylgalactosamine, N-acetylglucosamine, mannose, or fucose. Slower beta phase clearance may occur via renal glomerular filtration for molecules with an effective radius  $< 2$  nm (approximately 68 kD) and/or specific or non-specific uptake and metabolism in tissues. GlycoPEGylation may cap terminal sugars (e.g. galactose or N-acetylgalactosamine) and thereby block rapid alpha phase clearance via receptors that recognize these sugars. It may also confer a larger effective radius and thereby decrease the volume of distribution and tissue uptake, thereby prolonging the late beta phase. Thus, the precise impact of glycoPEGylation on alpha phase and beta phase half-lives will vary depending upon the size, state of glycosylation, and other parameters, as is well known in the art. Further explanation of "half-life" is found in Pharmaceutical Biotechnology (1997, DFA Crommelin and RD Sindelar, eds., Harwood Publishers, Amsterdam, pp 101 – 120).

The term "residence time", as used herein in the context of administering a peptide drug to a patient, is defined as the average time that drug stays in the body of the patient after dosing.

An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has  
5 been separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from  
10 other components which naturally accompany the nucleic acid, e.g., RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant  
15 DNA which is part of a hybrid nucleic acid encoding additional peptide sequence.

A "polynucleotide" means a single strand or parallel and anti-parallel strands of a nucleic acid. Thus, a polynucleotide may be either a single-stranded or a double-stranded nucleic acid.

The term "nucleic acid" typically refers to large polynucleotides. The term  
20 "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides.

Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction. The direction of  
25 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand which are located 5' to a reference point on the DNA are referred to as "upstream sequences"; sequences on the DNA strand which are 3' to a reference point on the DNA are referred to as "downstream sequences."

30 "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of

other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a nucleic acid sequence encodes a protein if transcription and translation of mRNA corresponding to that nucleic acid produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that nucleic acid or cDNA.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two peptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGGC share 50% homology.

As used herein, "homology" is used synonymously with "identity."

The determination of percent identity between two nucleotide or amino acid sequences can be accomplished using a mathematical algorithm. For example, a mathematical algorithm useful for comparing two sequences is the algorithm of Karlin and Altschul (1990, Proc. Natl. Acad. Sci. USA 87:2264-2268), modified as in Karlin and Altschul (1993, Proc. Natl. Acad. Sci. USA 90:5873-5877). This algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990, J. Mol. Biol. 215:403-410), and can be accessed, for example at the National Center for Biotechnology Information

(NCBI) world wide web site having the universal resource locator

"<http://www.ncbi.nlm.nih.gov/BLAST/>". BLAST nucleotide searches can be performed with the NBLAST program (designated "blastn" at the NCBI web site), using the following parameters: gap penalty = 5; gap extension penalty = 2; mismatch penalty = 3; match reward = 1; expectation value 10.0; and word size = 11 to obtain nucleotide sequences homologous to a nucleic acid described herein. BLAST protein searches can be performed with the XBLAST program (designated "blastn" at the NCBI web site) or the NCBI "blastp" program, using the following parameters: expectation value 10.0, BLOSUM62 scoring matrix to obtain amino acid sequences homologous to a protein molecule described herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997, Nucleic Acids Res. 25:3389-3402). Alternatively, PSI-Blast or PHI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Id.) and relationships between molecules which share a common pattern. When utilizing BLAST, Gapped BLAST, PSI-Blast, and PHI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

A "heterologous nucleic acid expression unit" encoding a peptide is defined as a nucleic acid having a coding sequence for a peptide of interest operably linked to one or more expression control sequences such as promoters and/or repressor sequences wherein at least one of the sequences is heterologous, i. e., not normally found in the host cell.

By describing two polynucleotides as "operably linked" is meant that a single-stranded or double-stranded nucleic acid moiety comprises the two polynucleotides arranged within the nucleic acid moiety in such a manner that at least one of the two polynucleotides is able to exert a physiological effect by which it is characterized upon the other. By way of example, a promoter operably linked to the coding region of a nucleic acid is able to promote transcription of the coding region.

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the



promoter/regulator sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A "constitutive promoter is a promoter which drives expression of a gene to which it is operably linked, in a constant manner in a cell. By way of example, promoters which drive expression of cellular housekeeping genes are considered to be constitutive promoters.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an *in vitro* expression system. Expression vectors include all those known in the art, such as cosmids, plasmids

(e.g., naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide.

A "genetically engineered" or "recombinant" cell is a cell having one or more modifications to the genetic material of the cell. Such modifications are seen to include, but are not limited to, insertions of genetic material, deletions of genetic material and insertion of genetic material that is extrachromosomal whether such material is stably maintained or not.

A "peptide" is an oligopeptide, polypeptide, peptide, protein or glycoprotein. The use of the term "peptide" herein includes a peptide having a sugar molecule attached thereto when a sugar molecule is attached thereto.

As used herein, "native form" means the form of the peptide when produced by the cells and/or organisms in which it is found in nature. When the peptide is produced by a plurality of cells and/or organisms, the peptide may have a variety of native forms.

"Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a peptide. Additionally, unnatural

amino acids, for example,  $\beta$ -alanine, phenylglycine and homoarginine are also included.

Amino acids that are not nucleic acid-encoded may also be used in the present invention.

Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the

invention. All of the amino acids used in the present invention may be either the D - or L -

isomer thereof. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a system that expresses the peptide. For a general review, *see*, Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

The term "peptide conjugate," refers to species of the invention in which a peptide is conjugated with a modified sugar as set forth herein.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the

naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline,  $\gamma$ -

carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an  $\alpha$  carbon that is linked to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified

5 R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

As used herein, amino acids are represented by the full name thereof, by the three

10 letter code corresponding thereto, or by the one-letter code corresponding thereto, as indicated in the following Table 1:

**Table 1.** Amino acids, and the three letter and one letter codes.

	Full Name	Three-Letter Code	One-Letter Code
	Aspartic Acid	Asp	D
15	Glutamic Acid	Glu	E
	Lysine	Lys	K
	Arginine	Arg	R
	Histidine	His	H
	Tyrosine	Tyr	Y
20	Cysteine	Cys	C
	Asparagine	Asn	N
	Glutamine	Gln	Q
	Serine	Ser	S
	Threonine	Thr	T
25	Glycine	Gly	G
	Alanine	Ala	A
	Valine	Val	V
	Leucine	Leu	L
	Isoleucine	Ile	I
30	Methionine	Met	M
	Proline	Pro	P
	Phenylalanine	Phe	F
	Tryptophan	Trp	W

35 The present invention also provides for analogs of proteins or peptides which comprise a protein as identified above. Analogs may differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do

not affect sequence, or by both. For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

- 5                   glycine, alanine;
- valine, isoleucine, leucine;
- aspartic acid, glutamic acid;
- asparagine, glutamine;
- serine, threonine;
- 10               lysine, arginine;
- phenylalanine, tyrosine.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro*, chemical derivatization of peptides, e.g., acetylation, or carboxylation. Also included  
15 are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a peptide during its synthesis and processing or in further processing steps; e.g., by exposing the peptide to enzymes which affect glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

20               It will be appreciated, of course, that the peptides may incorporate amino acid residues which are modified without affecting activity. For example, the termini may be derivatized to include blocking groups, i.e. chemical substituents suitable to protect and/or stabilize the N- and C-termini from "undesirable degradation", a term meant to encompass any type of enzymatic, chemical or biochemical breakdown of the compound at its termini  
25 which is likely to affect the function of the compound, i.e. sequential degradation of the compound at a terminal end thereof.

Blocking groups include protecting groups conventionally used in the art of peptide chemistry which will not adversely affect the *in vivo* activities of the peptide. For example, suitable N-terminal blocking groups can be introduced by alkylation or acylation of the N-  
30 terminus. Examples of suitable N-terminal blocking groups include C<sub>1</sub>-C<sub>5</sub> branched or unbranched alkyl groups, acyl groups such as formyl and acetyl groups, as well as substituted

forms thereof, such as the acetamidomethyl (Acm), Fmoc or Boc groups. Desamino analogs of amino acids are also useful N-terminal blocking groups, and can either be coupled to the N-terminus of the peptide or used in place of the N-terminal residue. Suitable C-terminal blocking groups, in which the carboxyl group of the C-terminus is either incorporated or not, include esters, ketones or amides. Ester or ketone-forming alkyl groups, particularly lower alkyl groups such as methyl, ethyl and propyl, and amide-forming amino groups such as primary amines ( $-NH_2$ ), and mono- and di-alkylamino groups such as methylamino, ethylamino, dimethylamino, diethylamino, methylethylamino and the like are examples of C-terminal blocking groups. Descarboxylated amino acid analogues such as agmatine are also useful C-terminal blocking groups and can be either coupled to the peptide's C-terminal residue or used in place of it. Further, it will be appreciated that the free amino and carboxyl groups at the termini can be removed altogether from the peptide to yield desamino and descarboxylated forms thereof without affect on peptide activity.

Other modifications can also be incorporated without adversely affecting the activity and these include, but are not limited to, substitution of one or more of the amino acids in the natural *L*-isomeric form with amino acids in the *D*-isomeric form. Thus, the peptide may include one or more *D*-amino acid residues, or may comprise amino acids which are all in the *D*-form. Retro-inverso forms of peptides in accordance with the present invention are also contemplated, for example, inverted peptides in which all amino acids are substituted with *D*-amino acid forms.

Acid addition salts of the present invention are also contemplated as functional equivalents. Thus, a peptide in accordance with the present invention treated with an inorganic acid such as hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, and the like, or an organic acid such as an acetic, propionic, glycolic, pyruvic, oxalic, malic, malonic, succinic, maleic, fumaric, tartaric, citric, benzoic, cinnamic, mandelic, methanesulfonic, ethanesulfonic, *p*-toluenesulfonic, salicylic and the like, to provide a water soluble salt of the peptide is suitable for use in the invention.

Also included are peptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such peptides include those containing residues other than naturally occurring *L*-amino

acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

As used herein, the term "MALDI" is an abbreviation for Matrix Assisted Laser Desorption Ionization. During ionization, SA-PEG (sialic acid-poly(ethylene glycol)) can be partially eliminated from the N-glycan structure of the glycoprotein.

As used herein, the term "glycosyltransferase," refers to any enzyme/protein that has the ability to transfer a donor sugar to an acceptor moiety.

As used herein, the term "modified sugar," refers to a naturally- or non-naturally- occurring carbohydrate that is enzymatically added onto an amino acid or a glycosyl residue of a peptide in a process of the invention. The modified sugar is selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and tri-phosphates), activated sugars (e.g., glycosyl halides, glycosyl mesylates) and sugars that are neither activated nor nucleotides.

The "modified sugar" is covalently functionalized with a "modifying group." Useful modifying groups include, but are not limited to, water-soluble polymers, therapeutic moieties, diagnostic moieties, biomolecules and the like. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a peptide.

The term "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences or be composed of a single amino acid, e.g. poly(lysine). Similarly, saccharides can be of mixed sequence or composed of a single saccharide subunit, e.g., dextran, amylose, chitosan, and poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol). Poly(ethylene imine) is an exemplary polyamine, and poly(aspartic) acid is a representative poly(carboxylic acid).

"Poly(alkylene oxide)" refers to a genus of compounds having a polyether backbone. Poly(alkylene oxide) species of use in the present invention include, for example, straight- and branched-chain species. Moreover, exemplary poly(alkylene oxide) species can terminate in one or more reactive, activatable, or inert groups. For example, poly(ethylene

glycol) is a poly(alkylene oxide) consisting of repeating ethylene oxide subunits, which may or may not include additional reactive, activatable or inert moieties at either terminus. Useful poly(alkylene oxide) species include those in which one terminus is "capped" by an inert group, e.g., monomethoxy-poly(alkylene oxide). When the molecule is a branched species, it may include multiple reactive, activatable or inert groups at the termini of the alkylene oxide chains and the reactive groups may be either the same or different. Derivatives of straight-chain poly(alkylene oxide) species that are heterobifunctional are also known in the art.

The term, "glycosyl linking group," as used herein refers to a glycosyl residue to which an agent (e.g., water-soluble polymer, therapeutic moiety, biomolecule) is covalently attached. In the methods of the invention, the "glycosyl linking group" becomes covalently attached to a glycosylated or unglycosylated peptide, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide. A "glycosyl linking group" is generally derived from a "modified sugar" by the enzymatic attachment of the "modified sugar" to an amino acid and/or glycosyl residue of the peptide. More specifically, a "glycosyl linking group," as used herein, refers to a moiety that covalently joins a "modifying group," as discussed herein, and an amino acid residue of a peptide. The glycosyl linking group-modifying group adduct has a structure that is a substrate for an enzyme. The enzymes for which the glycosyl linking group-modifying group adduct are substrates are generally those capable of transferring a saccharyl moiety onto an amino acid residue of a peptide, e.g., a glycosyltransferase, amidase, glycosidase, trans-sialidase, etc. The "glycosyl linking group" is interposed between, and covalently joins a "modifying group" and an amino acid residue of a peptide.

An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in which the individual saccharide monomer that links the conjugate is not degraded, e.g., oxidized, e.g., by sodium metaperiodate. "Intact glycosyl linking groups" of the invention may be derived from a naturally occurring oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure. An exemplary "intact glycosyl linking group" includes at least one intact, e.g., non-degraded, saccharyl moiety that is covalently attached to an amino acid residue on a peptide. The remainder of the "linking group" can have substantially any structure. For example, the modifying group is optionally linked directly to the intact saccharyl moiety. Alternatively,

the modifying group is linked to the intact saccharyl moiety via a linker arm. The linker arm can have substantially any structure determined to be useful in the selected embodiment. In an exemplary embodiment, the linker arm is one or more intact saccharyl moieties, i.e. "the intact glycosyl linking group" resembles an oligosaccharide. Another exemplary intact glycosyl linking group is one in which a saccharyl moiety attached, directly or indirectly, to the intact saccharyl moiety is degraded and derivatized (e.g., periodate oxidation followed by reductive amination). Still a further linker arm includes the modifying group attached to the intact saccharyl moiety, directly or indirectly, via a cross-linker, such as those described herein or analogues thereof.

"Degradation," as used herein refers to the removal of one or more carbon atoms from a saccharyl moiety.

The terms "targeting moiety" and "targeting agent", as used herein, refer to species that will selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art.

As used herein, "therapeutic moiety" means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is linked to a carrier, e.g., multivalent agents. Therapeutic moiety also includes peptides, and constructs that include peptides. Exemplary peptides include those disclosed in Figure 28 and Tables 6 and 7, herein. "Therapeutic moiety" thus means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is linked to a carrier, e.g., multivalent agents.

As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents,



anthracyclines, antibiotics, antimetabolic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, interferons and radioactive agents. Also encompassed within the scope of the term "anti-tumor drug," are conjugates of peptides with anti-tumor activity, e.g. TNF- $\alpha$ . Conjugates include, but are not limited to those formed between a therapeutic protein and a glycoprotein of the invention. A representative conjugate is that formed between PSGL-1 and TNF- $\alpha$ .

As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracenedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diphtheria toxin, and snake venom (e.g., cobra venom).

As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60 and technetium. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (e.g. EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, *etc.*). See, for example, Pitt *et al.*, "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge, 1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. See, for

example, Meares *et al.*, "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS," Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al.*, *Bioconjugate Chem.*, 9: 108-117 (1998); Song *et al.*, *Bioconjugate Chem.*, 8: 249-255 (1997).

As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the activity of the conjugate activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, intranasal or subcutaneous administration, intrathecal administration, or the implantation of a slow-release device *e.g.*, a mini-osmotic pump, to the subject.

The term "isolated" refers to a material that is substantially or essentially free from components, which are used to produce the material. For peptide conjugates of the invention, the term "isolated" refers to material that is substantially or essentially free from components, which normally accompany the material in the mixture used to prepare the peptide conjugate. "Isolated" and "pure" are used interchangeably. Typically, isolated peptide conjugates of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

When the peptide conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about

92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

Purity is determined by any art-recognized method of analysis (*e.g.*, band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

5       “Commercial scale” as used herein means about one or more gram of final product produced in the method.

10       “Essentially each member of the population,” as used herein, describes a characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified sugars added to a peptide are added to multiple, identical acceptor sites on the peptide. “Essentially each member of the population” speaks to the “homogeneity” of the sites on the peptide conjugated to a modified sugar and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and more preferably at least about 95% homogenous.

15       “Homogeneity,” refers to the structural consistency across a population of acceptor moieties to which the modified sugars are conjugated. Thus, in a peptide conjugate of the invention in which each modified sugar moiety is conjugated to an acceptor site having the same structure as the acceptor site to which every other modified sugar is conjugated, the peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates  
20 is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

25       When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more methods known to those of skill in the art, *e.g.*, liquid chromatography-mass spectrometry (LC-MS), matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF), capillary electrophoresis, and the like.

30       “Substantially uniform glycoform” or a “substantially uniform glycosylation pattern,” when referring to a glycopeptide species, refers to the percentage of acceptor moieties that

are glycosylated by the glycosyltransferase of interest (*e.g.*, fucosyltransferase). For example, in the case of a  $\alpha$ 1,2 fucosyltransferase, a substantially uniform fucosylation pattern exists if substantially all (as defined below) of the Gal $\beta$ 1,4-GlcNAc-R and sialylated analogues thereof are fucosylated in a peptide conjugate of the invention. It will be understood by one of skill in the art, that the starting material may contain glycosylated acceptor moieties (*e.g.*, fucosylated Gal $\beta$ 1,4-GlcNAc-R moieties). Thus, the calculated percent glycosylation will include acceptor moieties that are glycosylated by the methods of the invention, as well as those acceptor moieties already glycosylated in the starting material.

The term "substantially" in the above definitions of "substantially uniform" generally means at least about 40%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

## Description of the Invention

### I. Method to Remodel Glycan Chains

The present invention includes methods and compositions for the *in vitro* addition and/or deletion of sugars to or from a glycopeptide molecule in such a manner as to provide a peptide molecule having a specific customized or desired glycosylation pattern, preferably including the addition of a modified sugar thereto. A key feature of the invention therefore is to take a peptide produced by any cell type and generate a core glycan structure on the peptide, following which the glycan structure is then remodeled *in vitro* to generate a peptide having a glycosylation pattern suitable for therapeutic use in a mammal.

The importance of the glycosylation pattern of a peptide is well known in the art as are the limitations of present *in vivo* methods for the production of properly glycosylated peptides, particularly when these peptides are produced using recombinant DNA methodology. Moreover, until the present invention, it has not been possible to generate glycopeptides having a desired glycan structure thereon, wherein the peptide can be produced at industrial scale.

In the present invention, a peptide produced by a cell is enzymatically treated *in vitro* by the systematic addition of the appropriate enzymes and substrates therefor, such that sugar

moieties that should not be present on the peptide are removed, and sugar moieties, optionally including modified sugars, that should be added to the peptide are added in a manner to provide a glycopeptide having "desired glycosylation", as defined elsewhere herein.

A. Method to remodel N-linked glycans

5 In one aspect, the present invention takes advantage of the fact that most peptides of commercial or pharmaceutical interest comprise a common five sugar structure referred to herein as the trimannosyl core, which is N-linked to asparagine at the sequence Asn-X-Ser/Thr on a peptide chain. The elemental trimannosyl core consists essentially of two N-acetylglucosamine (GlcNAc) residues and three mannose (Man) residues attached to a  
10 peptide, i.e., it comprises these five sugar residues and no additional sugars, except that it may optionally include a fucose residue. The first GlcNAc is attached to the amide group of the asparagine and the second GlcNAc is attached to the first via a  $\beta$ 1,4 linkage. A mannose residue is attached to the second GlcNAc via a  $\beta$ 1,4 linkage and two mannose residues are attached to this mannose via an  $\alpha$ 1,3 and an  $\alpha$ 1,6 linkage respectively. A schematic depiction  
15 of a trimannosyl core structure is shown in Figure 1, left side. While it is the case that glycan structures on most peptides comprise other sugars in addition to the trimannosyl core, the trimannosyl core structure represents an essential feature of N-linked glycans on mammalian peptides.

The present invention includes the generation of a peptide having a trimannosyl core  
20 structure as a fundamental element of the structure of the glycan molecules contained thereon. Given the variety of cellular systems used to produce peptides, whether the systems are themselves naturally occurring or whether they involve recombinant DNA methodology, the present invention provides methods whereby a glycan molecule on a peptide produced in any cell type can be reduced to an elemental trimannosyl core structure. Once the elemental  
25 trimannosyl core structure has been generated then it is possible using the methods described herein, to generate *in vitro*, a desired glycan structure on the peptide which confers on the peptide one or more properties that enhances the therapeutic effectiveness of the peptide.

It should be clear from the discussion herein that the term "trimannosyl core" is used to describe the glycan structure shown in Figure 1, left side. Glycopeptides having a  
30 trimannosyl core structure may also have additional sugars added thereto, and for the most part, do have additional structures added thereto irrespective of whether the sugars give rise

to a peptide having a desired glycan structure. The term "elemental trimannosyl core structure" is defined elsewhere herein. When the term "elemental" is not included in the description of the "trimannosyl core structure," then the glycan comprises the trimannosyl core structure with additional sugars attached to the mannose sugars.

5       The term "elemental trimannosyl core glycopeptide" is used herein to refer to a glycopeptide having glycan structures comprised primarily of an elemental trimannosyl core structure. However, it may also optionally contain a fucose residue attached thereto. As discussed herein, elemental trimannosyl core glycopeptides are one optimal, and therefore preferred, starting material for the glycan remodeling processes of the invention.

10       Another optimal starting material for the glycan remodeling process of the invention is a glycan structure having a trimannosyl core wherein one or two additional GlcNAc residues are added to each of the  $\alpha$ 1,3 and the  $\alpha$ 1,6 mannose residues (see for example, the structure on the second line of Figure 2, second structure in from the left of the figure). This structure is referred to herein as "Man3GlcNAc4." When the structure is monoantennary, the  
15       structure is referred to herein as "Man3GlcNAc3." Optionally, this structure may also contain a core fucose molecule. Once the Man3GlcNAc3 or Man3GlcNAc4 structure has been generated then it is possible using the methods described herein, to generate *in vitro*, a desired glycan structure on the glycopeptide which confers on the glycopeptide one or more properties that enhances the therapeutic effectiveness of the peptide.

20       In their native form, the N-linked glycopeptides of the invention, and particularly the mammalian and human glycopeptides useful in the present invention, are N-linked glycosylated with a trimannosyl core structure and one or more sugars attached thereto.

25       The terms "glycopeptide" and "glycopolypeptide" are used synonymously herein to refer to peptide chains having sugar moieties attached thereto. No distinction is made herein to differentiate small glycopolypeptides or glycopeptides from large glycopolypeptides or glycopeptides. Thus, hormone molecules having very few amino acids in their peptide chain (e.g., often as few as three amino acids) and other much larger peptides are included in the general terms "glycopolypeptide" and "glycopeptide," provided they have sugar moieties attached thereto. However, the use of the term "peptide" does not preclude that peptide from  
30       being a glycopeptide.

An example of an N-linked glycopeptide having desired glycosylation is a peptide having an N-linked glycan having a trimannosyl core with at least one GlcNAc residue attached thereto. This residue is added to the trimannosyl core using N-acetyl glucosaminyltransferase I (GnT-I). If a second GlcNAc residue is added, N-acetyl glucosaminyltransferase II (GnT-II) is used. Optionally, additional GlcNAc residues may be added with GnT-IV and/or GnT-V, and a third bisecting GlcNAc residue may be attached to the  $\beta$ 1,4 mannose of the trimannosyl core using N-acetyl glucosaminyltransferase III (GnT-III). Optionally, this structure may be extended by treatment with  $\beta$ 1,4 galactosyltransferase to add a galactose residue to each non-bisecting GlcNAc, and even further optionally, using  $\alpha$ 2,3 or  $\alpha$ 2,6-sialyltransferase enzymes, sialic acid residues may be added to each galactose residue. The addition of a bisecting GlcNAc to the glycan is not required for the subsequent addition of galactose and sialic acid residues; however, with respect to the substrate affinity of the rat and human GnT-III enzymes, the presence of one or more of the galactose residues on the glycan precludes the addition of the bisecting GlcNAc in that the galactose-containing glycan is not a substrate for these forms of GnT-III. Thus, in instances where the presence of the bisecting GlcNAc is desired and these forms of GnT-III are used, it is important should the glycan contain added galactose and/or sialic residues, that they are removed prior to the addition of the bisecting GlcNAc. Other forms of GnT-III may not require this specific order of substrates for their activity. In the more preferred reaction, a mixture of GnT-I, GnT-II and GnT-III is added to the reaction mixture so that the GlcNAc residues can be added in any order.

Examples of glycan structures which represent the various aspects of peptides having "desired glycosylation" are shown in the drawings provided herein. The precise procedures for the *in vitro* generation of a peptide having "desired glycosylation" are described elsewhere herein. However, the invention should in no way be construed to be limited solely to any one glycan structure disclosed herein. Rather, the invention should be construed to include any and all glycan structures which can be made using the methodology provided herein.

In some cases, an elemental trimannosyl core alone may constitute the desired glycosylation of a peptide. For example, a peptide having only a trimannosyl core has been

shown to be a useful component of an enzyme employed to treat Gaucher disease (Mistry et al., 1966, Lancet 348: 1555-1559; Bijsterbosch et al., 1996, Eur. J. Biochem. 237:344-349) .

According to the present invention, the following procedures for the generation of peptides having desired glycosylation become apparent.

5 a) Beginning with a glycopeptide having one or more glycan molecules which have as a common feature a trimannosyl core structure and at least one or more of a heterogeneous or homogeneous mixture of one or more sugars added thereto, it is possible to increase the proportion of glycopeptides having an elemental trimannosyl core structure as the sole glycan structure or which have Man3GlcNAc3 or Man3GlcNAc4 as the sole glycan structure. This  
10 is accomplished *in vitro* by the systematic addition to the glycopeptide of an appropriate number of enzymes in an appropriate sequence which cleave the heterogeneous or homogeneous mixture of sugars on the glycan structure until it is reduced to an elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structure. Specific examples of how this is accomplished will depend on a variety of factors including in large part the type of cell  
15 in which the peptide is produced and therefore the degree of complexity of the glycan structure(s) present on the peptide initially produced by the cell. Examples of how a complex glycan structure can be reduced to an elemental trimannosyl core or a Man3GlcNAc3 or Man3GlcNAc4 structure are presented in Figure 2 or are described in detail elsewhere herein.

b) It is possible to generate a peptide having an elemental trimannosyl core structure  
20 as the sole glycan structure on the peptide by isolating a naturally occurring cell whose glycosylation machinery produces such a peptide. DNA encoding a peptide of choice is then transfected into the cell wherein the DNA is transcribed, translated and glycosylated such that the peptide of choice has an elemental trimannosyl core structure as the sole glycan structure thereon. For example, a cell lacking a functional GnT-I enzyme will produce several types of  
25 glycopeptides. In some instances, these will be glycopeptides having no additional sugars attached to the trimannosyl core. However, in other instances, the peptides produced may have two additional mannose residues attached to the trimannosyl core, resulting in a Man5 glycan. This is also a desired starting material for the remodeling process of the present invention. Specific examples of the generation of such glycan structures are described  
30 herein.



c) Alternatively, it is possible to genetically engineer a cell to confer upon it a specific glycosylation machinery such that a peptide having an elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structure as the sole glycan structure on the peptide is produced. DNA encoding a peptide of choice is then transfected into the cell wherein the DNA is transcribed, translated and glycosylated such that the peptide of choice has an increased number of glycans comprising solely an elemental trimannosyl core structure. For example, certain types of cells that are genetically engineered to lack GnT-I, may produce a glycan having an elemental trimannosyl core structure, or, depending on the cell, may produce a glycan having a trimannosyl core plus two additional mannose residues attached thereto (Man5). When the cell produces a Man5 glycan structure, the cell may be further genetically engineered to express mannosidase 3 which cleaves off the two additional mannose residues to generate the trimannosyl core. Alternatively, the Man5 glycan may be incubated *in vitro* with mannosidase 3 to have the same effect.

d) When a peptide is expressed in an insect cell, the glycan on the peptide comprises a partially complex chain. Insect cells also express hexosaminidase in the cells which trims the partially complex chain back to a trimannosyl core structure which can then be remodeled as described herein.

e) It is readily apparent from the discussion in b), c) and d) that it is not necessary that the cells produce only peptides having elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structures attached thereto. Rather, unless the cells described in b) and c) produce peptides having 100% elemental trimannosyl core structures (i.e., having no additional sugars attached thereto) or 100% of Man3GlcNAc3 or Man3GlcNAc4 structures, the cells in fact produce a heterogeneous mixture of peptides having, in combination, elemental trimannosyl core structures, or Man3GlcNAc3 or Man3GlcNAc4 structures, as the sole glycan structure in addition to these structures having additional sugars attached thereto. The proportion of peptides having a trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structures having additional sugars attached thereto, as opposed to those having one structure, will vary depending on the cell which produces them. The complexity of the glycans (i.e. which and how many sugars are attached to the trimannosyl core) will also vary depending on the cell which produces them.

f) Once a glycopeptide having an elemental trimannosyl core or a trimannosyl core with one or two GlcNAc residues attached thereto is produced by following a), b) or c) above, according to the present invention, additional sugar molecules are added *in vitro* to the trimannosyl core structure to generate a peptide having desired glycosylation (i.e., a peptide  
5 having an *in vitro* customized glycan structure).

g) However, when it is the case that a peptide having an elemental trimannosyl core or Man3GlcNAc4 structure with some but not all of the desired sugars attached thereto is produced, then it is only necessary to add any remaining desired sugars without reducing the glycan structure to the elemental trimannosyl core or Man3GlcNAc4 structure. Therefore, in  
10 some cases, a peptide having a glycan structure having a trimannosyl core structure with additional sugars attached thereto, will be a suitable substrate for remodeling.

#### Isolation of an elemental trimannosyl core glycopeptide

The elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 glycopeptides of  
15 the invention may be isolated and purified, if necessary, using techniques well known in the art of peptide purification. Suitable techniques include chromatographic techniques, isoelectric focusing techniques, ultrafiltration techniques and the like. Using any such techniques, a composition of the invention can be prepared in which the glycopeptides of the invention are isolated from other peptides and from other components normally found within  
20 cell culture media. The degree of purification can be, for example, 90% with respect to other peptides or 95%, or even higher, e.g., 98%. See, e.g., Deutscher et al. (ed., 1990, Guide to Protein Purification, Harcourt Brace Jovanovich, San Diego).

The heterogeneity of N-linked glycans present in the glycopeptides produced by the prior art methodology generally only permits the isolation of a small portion of the target  
25 glycopeptides which can be modified to produce desired glycopeptides. In the present methods, large quantities of elemental trimannosyl core glycopeptides and other desired glycopeptides, including Man3GlcNAc3 or Man3GlcNAc4 glycans, can be produced which can then be further modified to generate large quantities of peptides having desired glycosylation.

Specific enrichment of any particular type of glycan linked to a peptide may be accomplished using lectins which have an affinity for the desired glycan. Such techniques are well known in the art of glycobiology.

5 A key feature of the invention which is described in more detail below, is that once a core glycan structure is generated on any peptide, the glycan structure is then remodeled *in vitro* to generate a peptide having desired glycosylation that has improved therapeutic use in a mammal. The mammal may be any type of suitable mammal, and is preferably a human.

The various scenarios and the precise methods and compositions for generating peptides with desired glycosylation will become evident from the disclosure which follows.

10 The ultimate objective of the production of peptides for therapeutic use in mammals is that the peptides should comprise glycan structures that facilitate rather than negate the therapeutic benefit of the peptide. As disclosed throughout the present specification, peptides produced in cells may be treated *in vitro* with a variety of enzymes which catalyze the cleavage of sugars that should not be present on the glycan and the addition of sugars which  
15 should be present on the glycan such that a peptide having desired glycosylation and thus suitable for therapeutic use in mammals is generated. The generation of different glycoforms of peptides in cells is described above. A variety of mechanisms for the generation of peptides having desired glycosylation is now described, where the starting material i.e., the peptide produced by a cell may differ from one cell type to another. As will become apparent  
20 from the present disclosure, it is not necessary that the starting material be uniform with respect to its glycan composition. However, it is preferable that the starting material be enriched for certain glycoforms in order that large quantities of end product, i.e., correctly glycosylated peptides are produced.

In a preferred embodiment according to the present invention, the degradation and  
25 synthesis events that result in a peptide having desired glycosylation involve at some point, the generation of an elemental trimannosyl core structure or a Man3GlcNAc3 or Man3GlcNAc4 structure on the peptide.

The present invention also provides means of adding one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one of the selected  
30 glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not

present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a peptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the peptide. See for example WO 98/31826.

Addition or removal of any carbohydrate moieties present on the peptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the peptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin et al., 1987, Arch. Biochem. Biophys. 259: 52 and by Edge et al., 1981, Anal. Biochem. 118: 131. Enzymatic cleavage of carbohydrate moieties on peptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., 1987, Meth. Enzymol. 138: 350.

Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified sugars used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent No. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) sites for N- and O-glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Dealing specifically with the examples shown in several of the figures provided herein, a description of the sequence of *in vitro* enzymatic reactions for the production of desired glycan structures on peptides is now presented. The precise reaction conditions for

each of the enzymatic conversions disclosed below are well known to those skilled in the art of glycobiology and are therefore not repeated here. For a review of the reaction conditions for these types of reactions, see Sadler et al., 1982, *Methods in Enzymology* 83:458-514 and references cited therein.

5 In Figure 1 there is shown the structure of an elemental trimannosyl core glycan on the left side. It is possible to convert this structure to a complete glycan structure having a bisecting GlcNAc by incubating the elemental trimannosyl core structure in the presence of GnT-I, followed by GnT-II, and further followed by GnT-III, and a sugar donor comprising UDP-GlcNAc, wherein GlcNAc is sequentially added to the elemental trimannosyl core  
10 structure to generate a trimannosyl core having a bisecting GlcNAc. In some instances, for example when remodeling Fc glycans as described herein, the order of addition of GnT-I, GnT-II and GnT-III may be contrary to that reported in the literature. The bisecting GlcNAc structure may be produced by adding a mixture of GnT-I, GnT-II and GnT-III and UDP-GlcNAc to the reaction mixture

15 In Figure 3 there is shown the conversion of a bisecting GlcNAc containing trimannosyl core glycan to a complex glycan structure comprising galactose and N-acetyl neuraminic acid. The bisecting GlcNAc containing trimannosyl core glycan is first incubated with galactosyltransferase and UDP-Gal as a donor molecule, wherein two galactose residues are added to the peripheral GlcNAc residues on the molecule. The enzyme NeuAc-  
20 transferase is then used to add two NeuAc residues one to each of the galactose residues.

In Figure 4 there is shown the conversion of a high mannose glycan structure to an elemental trimannosyl core glycan. The high mannose glycan (Man9) is incubated sequentially in the presence of the mannosidase 1 to generate a Man5 structure and then in the presence of mannosidase 3, wherein all but three mannose residues are removed from the  
25 glycan. Alternatively, incubation of the Man9 structure may be trimmed back to the trimannosyl core structure solely by incubation in the presence of mannosidase 3. According to the schemes presented in Figures 1 and 3 above, conversion of this elemental trimannosyl core glycan to a complex glycan molecule is then possible.

30 In Figure 5 there is shown a typical complex N-linked glycan structure produced in plant cells. It is important to note that when plant cells are deficient in GnT-I enzymatic activity, xylose and fucose cannot be added to the glycan. Thus, the use of GnT-I knock-out

cells provides a particular advantage in the present invention in that these cells produce peptides having an elemental trimannosyl core onto which additional sugars can be added without performing any "trimming back" reactions. Similarly, in instances where the structure produced in a plant cell may be of the Man5 variety of glycan, if GnT-I is absent in these cells, xylose and fucose cannot be added to the structure. In this case, the Man5 structure may be trimmed back to an elemental trimannosyl core (Man3) using mannosidase 3. According to the methods provided herein, it is now possible to add desired sugar moieties to the trimannosyl core to generate a desired glycan structure.

In Figure 6 there is shown a typical complex N-linked glycan structure produced in insect cells. As is evident, additional sugars, such as, for example, fucose may also be present. Further although not shown here, insect cells may produce high mannose glycans having as many as nine mannose residues and may have additional sugars attached thereto. It is also the case in insect cells that GnT-I knock out cells prevent the addition of fucose residues to the glycan. Thus, production of a peptide in insect cells may preferably be accomplished in a GnT-I knock out cell. The glycan thus produced may then be trimmed back *in vitro* if necessary using any of the methods and schemes described herein, and additional sugars may be added *in vitro* thereto also using the methods and schemes provided herein.

In Figure 2 there is shown glycan structures in various stages of completion. Specifically, the *in vitro* enzymatic generation of an elemental trimannosyl core structure from a complex carbohydrate glycan structure which does not contain a bisecting GlcNAc residue is shown. Also shown is the generation of a glycan structure therefrom which contains a bisecting GlcNAc. Several intermediate glycan structures which can be produced are shown. These structures can be produced by cells, or can be produced in the *in vitro* trimming back reactions described herein. Sugar moieties may be added *in vitro* to the elemental trimannosyl core structure, or to any suitable intermediate structure in order that a desired glycan is produced.

In Figure 7 there is shown a series of possible *in vitro* reactions which can be performed to trim back and add onto glycans beginning with a high mannose structure. For example, a Man9 glycan may be trimmed using mannosidase 1 to generate a Man5 glycan, or it may be trimmed to a trimannosyl core using mannosidase 3 or one or more microbial

mannosidases. GnT-I and or GnT-II may then be used to transfer additional GlcNAc residues onto the glycan. Further, there is shown the situation which would not occur when the glycan molecule is produced in a cell that does not have GnT-I (see shaded box). For example, fucose and xylose may be added to a glycan only when GnT-I is active and facilitates the transfer of a GlcNAc to the molecule.

Figure 8 depicts well known strategies for the synthesis of biantennary, triantennary and even tetraantennary glycan structures beginning with the trimannosyl core structure. According to the methods of the invention, it is possible to synthesize each of these structures *in vitro* using the appropriate enzymes and reaction conditions well known in the art of glycobiology.

Figure 9 depicts two methods for synthesis of a monoantennary glycan structure beginning from a high mannose (6 to 9 mannose moieties) glycan structures. A terminal sialic acid-PEG moiety may be added in place of the sialic acid moiety in accordance with glycoPEGylation methodology described herein. In the first method, endo-H is used to cleave the glycan structure on the peptide back to the first GlcNAc residue. Galactose is then added using galactosyltransferase and sialylated-PEG is added as described elsewhere herein. In the second method, mannosidase I is used to cleave mannose residues from the glycan structure in the peptide. A galactose residue is added to one arm of the remaining mannose residues which were cleaved off the glycan using Jack Bean  $\alpha$ -mannosidase. Sialylated-PEG is then added to this structure as directed.

Figure 10 depicts two additional methods for synthesis of a monoantennary glycan structures beginning from high mannose (6 to 9 mannose moieties) glycan structure. As in Figure 9, a terminal sialic acid-PEG moiety may be added in place of the sialic acid moiety in accordance with the glycoPEGylation methodology described herein. In the situation described here, some of the mannose residues from the arm to which sialylated-PEG is not added, are removed.

In Figure 11 there is shown a scheme for the synthesis of yet more complex carbohydrate structures beginning with a trimannosyl core structure. For example, a scheme for the *in vitro* production of Lewis x and Lewis a antigen structures, which may or may not be sialylated is shown. Such structures when present on a peptide may confer on the peptide immunological advantages for upregulating or downregulating the immune response. In

addition, such structures are useful for targeting the peptide to specific cells, in that these types of structures are involved in binding to cell adhesion peptides and the like.

Figure 12 is an exemplary scheme for preparing an array of O-linked peptides originating with serine or threonine.

5        Figure 13 is a series of diagrams depicting the four types of O-linked glycan structure termed cores 1 through 4. The core structure is outlined in dotted lines. Sugars which may also be included in this structure include sialic acid residues added to the galactose residues, and fucose residues added to the GlcNAc residues.

10        Thus, in preferred embodiments, the present invention provides a method of making an N-linked glycosylated glycopeptide by providing an isolated and purified glycopeptide to which is attached an elemental trimannosyl core or a Man3GlcNAc4 structure, contacting the glycopeptide with a glycosyltransferase enzyme and a donor molecule having a glycosyl moiety under conditions suitable to transfer the glycosyl moiety to the glycopeptide. Customization of a trimannosyl core glycopeptide or Man3GlcNAc4 glycopeptide to produce  
15 a peptide having a desired glycosylation pattern is then accomplished by the sequential addition of the desired sugar moieties, using techniques well known in the art.

#### Determination of Glycan Primary Structure

When an N-linked glycopeptide is produced by a cell, as noted elsewhere herein, it may comprise a heterogeneous mixture of glycan structures which must be reduced to a  
20 common, generally elemental trimannosyl core or Man3GlcNAc4 structure, prior to adding other sugar moieties thereto. In order to determine exactly which sugars should be removed from any particular glycan structure, it is sometimes necessary that the primary glycan structure be identified. Techniques for the determination of glycan primary structure are well known in the art and are described in detail, for example, in Montreuil, "Structure and  
25 Biosynthesis of Glycopeptides" In Polysaccharides in Medicinal Applications, pp. 273-327, 1996, Eds. Severian Damitriu, Marcel Dekker, NY. It is therefore a simple matter for one skilled in the art of glycobiology to isolate a population of peptides produced by a cell and determine the structure(s) of the glycans attached thereto. For example, efficient methods are available for (i) the splitting of glycosidic bonds either by chemical cleavage such as  
30 hydrolysis, acetolysis, hydrazinolysis, or by nitrous deamination; (ii) complete methylation followed by hydrolysis or methanolysis and by gas-liquid chromatography and mass



spectroscopy of the partially methylated monosaccharides; and (iii) the definition of anomeric linkages between monosaccharides using exoglycosidases, which also provide insight into the primary glycan structure by sequential degradation. In particular, the techniques of mass spectroscopy and nuclear magnetic resonance (NMR) spectrometry, especially high field

5 NMR have been successfully used to determine glycan primary structure.

Kits and equipment for carbohydrate analysis are also commercially available.

Fluorophore Assisted Carbohydrate Electrophoresis (FACE<sup>®</sup>) is available from Glyko, Inc. (Novato, CA). In FACE analysis, glycoconjugates are released from the peptide with either

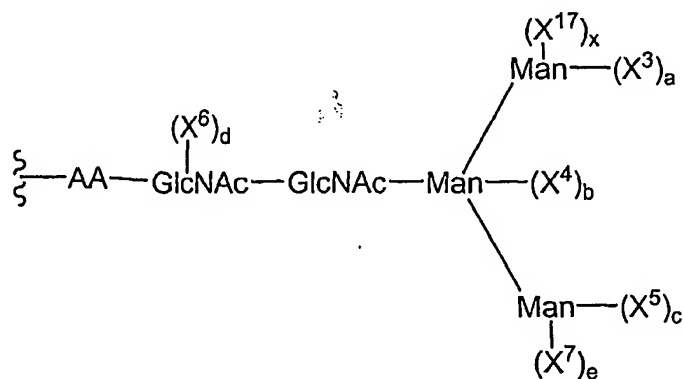
10 Endo H or N-glycanase (PNGase F) for N-linked glycans, or hydrazine for Ser/Thr linked glycans. The glycan is then labeled at the reducing end with a fluorophore in a non-structure discriminating manner. The fluorophore labeled glycans are then separated in polyacrylamide gels based on the charge/mass ratio of the saccharide as well as the hydrodynamic volume. Images are taken of the gel under UV light and the composition of the glycans are determined by the migration distance as compared with the standards.

15 Oligosaccharides can be sequenced in this manner by analyzing migration shifts due to the sequential removal of saccharides by exoglycosidase digestion.

#### Exemplary embodiment

The remodeling of N-linked glycosylation is best illustrated with reference to Formula

1:



20

where  $\text{X}^3$ ,  $\text{X}^4$ ,  $\text{X}^5$ ,  $\text{X}^6$ ,  $\text{X}^7$  and  $\text{X}^{17}$  are (independently selected) monosaccharide or oligosaccharide residues; and

a, b, c, d, e and x are (independently selected) 0, 1 or 2, with the proviso that at least one member selected from a, b, c, d, e and x are 1 or 2.

Formula 1 describes glycan structure comprising the tri-mannosyl core, which is preferably covalently linked to an asparagine residue on a peptide backbone. Preferred expression systems will express and secrete exogenous peptides with N-linked glycans comprising the tri-mannosyl core. Using the remodeling method of the invention, the glycan structures on these peptides can be conveniently remodeled to any glycan structure desired. Exemplary reaction conditions are found throughout the examples and in the literature.

In preferred embodiments, the glycan structures are remodeled so that the structure described in Formula 1 has specific determinates. The structure of the glycan can be chosen to enhance the biological activity of the peptide, give the peptide a new biological activity, remove the biological activity of peptide, or better approximate the glycosylation pattern of the native peptide, among others.

In the first preferred embodiment, the peptide N-linked glycans are remodeled to better approximate the glycosylation pattern of native human proteins. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:

$X^3$  and  $X^5 = \text{-GlcNAc-Gal-SA}$ ;

$a$  and  $c = 1$ ;

$d = 0$  or  $1$ ;

$b, e$  and  $x = 0$ .

This embodiment is particularly advantageous for human peptides expressed in heterologous cellular expression systems. By remodeling the N-linked glycan structures to this configuration, the peptide can be made less immunogenic in a human patient, and/or more stable, among others.

In the second preferred embodiment, the peptide N-linked glycans are remodeled to have a bisecting GlcNAc residue on the tri-mannosyl core. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:

$X^3$  and  $X^5$  are  $\text{-GlcNAc-Gal-SA}$ ;

$a$  and  $c = 1$ ;

$X^4$  is GlcNAc;

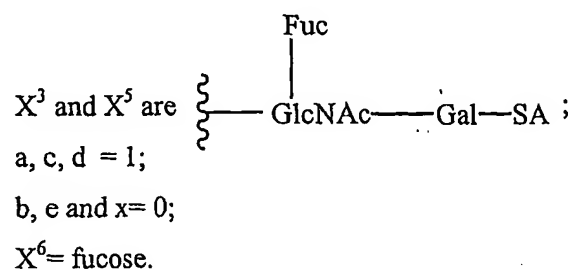
$b=1$ ;

$d = 0$  or  $1$ ;

e and x = 0.

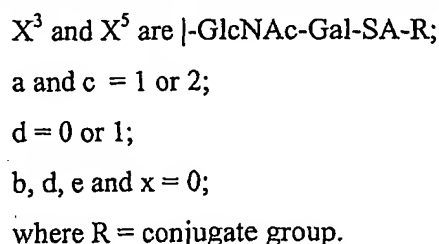
This embodiment is particularly advantageous for recombinant antibody molecules expressed in heterologous cellular systems. When the antibody molecule includes a Fc-mediated cellular cytotoxicity, it is known that the presence of bisected oligosaccharides linked the Fc domain dramatically increased antibody-dependent cellular cytotoxicity.

In a third preferred embodiment, the peptide N-linked glycans are remodeled to have a sialylated Lewis X moiety. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:



15 This embodiment is particularly advantageous when the peptide which is being remodeling is intended to be targeted to selectin molecules and cells exhibiting the same.

In a fourth preferred embodiment, the peptide N-linked glycans are remodeled to have a conjugated moiety. The conjugated moiety may be a PEG molecule, another peptide, a small molecule such as a drug, among others. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:



The conjugated moiety may be a PEG molecule, another peptide, a small molecule such as a drug, among others. This embodiment therefore is useful for conjugating the peptide to PEG molecules that will slow the clearance of the peptide from the patient's bloodstream, to peptides that will target both peptides to a specific tissue or cell, or to another peptide of complementary therapeutic use.

30

It will be clear to one of skill in the art that the invention is not limited to the preferred glycan molecules described above. The preferred embodiments are only a few of the many useful glycan molecules that can be made by the remodeling method of the invention. Those skilled in the art will know how to design other useful glycans.

5 In the first exemplary embodiments, the peptide is expressed in a CHO (Chinese hamster ovarian cell line) according to methods well known in the art. When a peptide with N-linked glycan consensus sites is expressed and secreted from CHO cells, the N-linked glycans will have the structures depicted in top row of Figure 2, but also comprising a core fucose. While all of these structures may be present, by far the most common structures are  
10 the two at the right side. In the terms of Formula 1,

$X^3$  and  $X^5$  are  $[-\text{GlcNAc-Gal}-(\text{SA})]$ ;

$a$  and  $c = 1$ ;

$b$ ,  $e$  and  $x = 0$ , and

$d = 0$  or  $1$ .

15 Therefore, in one exemplary embodiment, the N-linked glycans of peptides expressed in CHO cells are remodeled to the preferred humanized glycan by contacting the peptides with a glycosyltransferase that is specific for a galactose acceptor molecule and a sialic acid donor molecule. This process is illustrated in Figure 2 and Example 17. In another exemplary  
20 embodiment, the N-linked glycans of a peptide expressed and secreted from CHO cells are remodeled to be the preferred PEGylated structures. The peptide is first contacted with a glycosidase specific for sialic acid to remove the terminal SA moiety, and then contacted with a glycosyltransferase specific for a galactose acceptor moiety and an sialic acid acceptor moiety, in the presence of PEG- sialic acid-nucleotide donor molecules. Optionally, the  
25 peptide may then be contacted with a glycosyltransferase specific for a galactose acceptor moiety and an sialic acid acceptor moiety, in the presence of sialic acid-nucleotide donor molecules to ensure complete the SA capping of all of the glycan molecules.

In other exemplary embodiments, the peptide is expressed in insect cells, such as the sf9 cell line, according to methods well known in the art. When a peptide with N-linked glycan consensus sites is expressed and secreted from sf9 cells, the N-linked glycans will  
30 often have the structures depicted in top row of Figure 6. In the terms of Formula 1:

$X^3$  and  $X^5$  are  $[-\text{GlcNAc}]$ ;

a and c = 0 or 1;

b = 0;

X<sup>6</sup> is fucose,

d = 0, 1 or 2; and

5 e and x = 0.

The trimannose core is present in the vast majority of the N-linked glycans made by insect cells, and sometimes an antennary GlcNAc and/or fucose residue(s) are also present. Note that the glycan may have no core fucose, it may have a single core fucose having either linkage, or it may have a single core fucose with a preponderance of a single linkage. In one  
10 exemplary embodiment, the N-linked glycans of a peptide expressed and secreted from insect cells is remodeled to the preferred humanized glycan by first contacting the glycans with a glycosidase specific to fucose molecules, then contacting the glycans with a glycosyltransferases specific to the mannose acceptor molecule on each antennary of the trimannose core, a GlcNAc donor molecule in the presence of nucleotide-GlcNAc molecules;  
15 then contacting the glycans with a glycosyltransferase specific to a GlcNAc acceptor molecule, a Gal donor molecule in the presence of nucleotide-Gal molecules; and then contacting the glycans with a glycosyltransferase specific to a galactose acceptor molecule, a sialic acid donor molecule in the presence of nucleotide-SA molecules. One of skill in the art will appreciate that the fucose molecules, if any, can be removed at any time during the  
20 procedure, and if the core fucose is of the same alpha 1,6 linkage as found in human glycans, it may be left intact. In another exemplary embodiment, the humanized glycan of the previous example is remodeled further to the sialylated Lewis X glycan by contacting the glycan further with a glycosyltransferase specific to a GlcNAc acceptor molecule, a fucose donor molecule in the presence of nucleotide-fucose molecules. This process is illustrated in  
25 Figure 11 and Example 39.

In yet other exemplary embodiments, the peptide is expressed in yeast, such as *Saccharomyces cerevisiae*, according to methods well known in the art. When a peptide with N-linked glycan consensus sites is expressed and secreted from *S. cerevisiae* cells, the N-linked glycans will have the structures depicted at the left in Figure 4. The N-linked glycans  
30 will always have the trimannosyl core, which will often be elaborated with mannose or related polysaccharides of up to 1000 residues. In the terms of Formula 1:

$X^3$  and  $X^5 = \text{Man} - \text{Man} - (\text{Man})_{0-1000}$ ;

a and c = 1 or 2;

b, d, e and x = 0.

In one exemplary embodiment, the N-linked glycans of a peptide expressed and  
 5 secreted from yeast cells are remodeled to the elemental trimannose core by first contacting the glycans with a glycosidase specific to  $\alpha 2$  mannose molecules, then contacting the glycans with a glycosidase specific to  $\alpha 6$  mannose molecules. This process is illustrated in Figure 4 and Example 38.

In another exemplary embodiment, the N-linked glycans are further remodeled to  
 10 make a glycan suitable for an recombinant antibody with Fc-mediated cellular toxicity function by contacting the elemental trimannose core glycans with a glycosyltransferase specific to the mannose acceptor molecule on each antennary of the trimannose core and a GlcNAc donor molecule in the presence of nucleotide-GlcNAc molecules. Then, the glycans are contacted with a glycosyltransferase specific to the acceptor mannose molecule in the  
 15 middle of the trimannose core, a GlcNAc donor molecule in the presence of nucleotide-GlcNAc molecules and further contacting the glycans with a glycosyltransferase specific to a GlcNAc acceptor molecule, a Gal donor molecule in the presence of nucleotide-Gal molecules; and then optionally contacting the glycans with a glycosyltransferase specific to a galactose acceptor molecule and further optionally a sialic acid donor molecule in the  
 20 presence of nucleotide-SA molecules. This process is illustrated in Figures 1, 2 and 3.

In another exemplary embodiment, the peptide is expressed in bacterial cells, in particular *E. coli* cells, according to methods well known in the art. When a peptide with N-linked glycans consensus sites is expressed in *E. coli* cells, the N-linked consensus sites will not be glycosylated. In an exemplary embodiment, a humanized glycan molecule is built out  
 25 from the peptide backbone by contacting the peptides with a glycosyltransferase specific for a N-linked consensus site and a GlcNAc donor molecule in the presence of nucleotide-GlcNAc; and further sequentially contacting the growing glycans with glycosyltransferases specific for the acceptor and donor moieties in the present of the required donor moiety until the desired glycan structure is completed. When a peptide with N-linked glycans is  
 30 expressed in a eukaryotic cells but without the proper leader sequences that direct the nascent peptide to the golgi apparatus, the mature peptide is likely not to be glycosylated. In this case

as well the peptide may be given N-linked glycosylation by building out from the peptide N-linked consensus site as aforementioned. When a protein is chemically modified with a sugar moiety, it can be built out as aforementioned.

These examples are meant to illustrate the invention, and not to limit it. One of skill  
5 in the art will appreciate that the steps taken in each example may in some circumstances be able to be performed in a different order to get the same result. One of skill in the art will also understand that a different set of steps may also produce the same resulting glycan. The preferred remodeled glycan is by no means specific to the expression system that the peptide is expressed in. The remodeled glycans are only illustrative and one of skill in the art will  
10 know how to take the principles from these examples and apply them to peptides produced in different expression systems to make glycans not specifically described herein.

#### B. Method to remodel O-linked glycans

O-glycosylation is characterized by the attachment of a variety of monosaccharides in  
15 an O-glycosidic linkage to hydroxy amino acids. O-glycosylation is a widespread post-translational modification in the animal and plant kingdoms. The structural complexity of glycans O-linked to proteins vastly exceeds that of N-linked glycans. Serine or threonine residues of a newly translated peptide become modified by virtue of a peptidyl GalNAc transferase in the cis to trans compartments of the Golgi. The site of O-glycosylation is  
20 determined not only by the sequence specificity of the glycosyltransferase, but also epigenetic regulation mediated by competition between different substrate sites and competition with other glycosyltransferases responsible for forming the glycan.

The O-linked glycan has been arbitrarily defined as having three regions: the core, the backbone region and the peripheral region. The "core" region of an O-linked glycan is the  
25 inner most two or three sugars of the glycan chain proximal to the peptide. The backbone region mainly contributes to the length of the glycan chain formed by uniform elongation. The peripheral region exhibits a high degree of structural complexity. The structural complexity of the O-linked glycans begins with the core structure. In most cases, the first sugar residue added at the O-linked glycan consensus site is GalNAc; however the sugar may  
30 also be GlcNAc, glucose, mannose, galactose or fucose, among others. Figure 12 is a

diagram of some of the known O-linked glycan core structures and the enzymes responsible for their in vivo synthesis.

In mammalian cells, at least eight different O-linked core structures are found, all based on a core- $\alpha$ -GalNAc residue. The four core structures depicted in Figure 13 are the most common. Core 1 and core 2 are the most abundant structures in mammalian cells, and core 3 and core 4 are found in more restricted, organ-characteristic expression systems. O-linked glycans are reviewed in Montreuil, Structure and Synthesis of Glycopeptides, In Polysaccharides in Medicinal Applications, pp. 273-327, 1996, Eds. Severian Damitriu, Marcel Dekker, NY, and in Schachter and Brockhausen, The Biosynthesis of Branched O-Linked Glycans, 1989, Society for Experimental Biology, pp. 1-26 (Great Britain).

It will be apparent from the present disclosure that the glycan structure of O-glycosylated peptides can be remodeled using similar techniques to those described for N-linked glycans. O-glycans differ from N-glycans in that they are linked to a serine or threonine residue rather than an asparagine residue. As described herein with respect to N-glycan remodeling, hydrolytic enzymes can be used to cleave unwanted sugar moieties in an O-linked glycan and additional desired sugars can then be added thereto, to build a customized O-glycan structure on the peptide (See Figures 12 and 13).

The initial step in O-glycosylation in mammalian cells is the attachment of N-acetylgalactosamine (GalNAc) using any of a family of at least eleven known  $\alpha$ -N-acetylgalactosaminyltransferases, each of which has a restricted acceptor peptide specificity. Generally, the acceptor peptide recognized by each enzyme constitutes a sequence of at least ten amino acids. Peptides that contain the amino acid sequence recognized by one particular GalNAc-transferase become O-glycosylated at the acceptor site if they are expressed in a cell expressing the enzyme and if they are appropriately localized to the Golgi apparatus where UDP-GalNAc is also present.

However, in the case of recombinant proteins, the initial attachment of the GalNAc may not take place. The  $\alpha$ -N-acetylgalactosaminyltransferase enzyme native to the expressing cell may have a consensus sequence specificity which differs from that of the recombinant peptide being expressed.

The desired recombinant peptide may be expressed in a bacterial cell, such as *E. coli*, that does not synthesize glycan chains. In these cases, it is advantageous to add the initial



GalNAc moiety *in vitro*. The GalNAc moiety can be introduced *in vitro* onto the peptide once the recombinant peptide has been recovered in a soluble form, by contacting the peptide with the appropriate GalNAc transferase in the presence of UDP-GalNAc.

In one embodiment, an additional sequence of amino acids that constitute an effective  
5 acceptor for transfer of an O-linked sugar may be present. Such an amino acid sequence is encoded by a DNA sequence fused in frame to the coding sequence of the peptide, or alternatively, may be introduced by chemical means. The peptide may be otherwise lacking glycan chains. Alternately, the peptide may have N- and/or O-linked glycan chains but require an additional glycosylation site, for example, when an additional glycan substituent is  
10 desired.

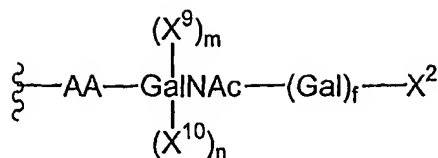
In an exemplary embodiment, the amino acid sequence PTTTK-COOH, which is the natural GalNAc acceptor sequence in the human mucin MUC-1, is added as a fusion tag. The fusion protein is then expressed in *E. coli* and purified. The peptide is then contacted with recombinant human GalNAc-transferases T3 or T6 in the presence of UDP-GalNAc to  
15 transfer a GalNAc residue onto the peptide *in vitro*.

This glycan chain on the peptide may then be further elongated using the methods described in reference to the N-linked or O-linked glycans herein. Alternatively, the GalNAc transferase reaction can be carried out in the presence of UDP-GalNAc to which PEG is covalently substituted in the O-3, 4, or 6 positions or the N-2 position. Glycoconjugation is  
20 described in detail elsewhere herein. Any antigenicity introduced into the peptide by the new peptide sequence can be conveniently masked by PEGylation of the associated glycan. The acceptor site fusion technique can be used to introduce not only a PEG moiety, but to introduce other glycan and non-glycan moieties, including, but not limited to, toxins, anti-infectives, cytotoxic agents, chelators for radionucleotides, and glycans with other  
25 functionalities, such as tissue targeting.

#### Exemplary Embodiments

The remodeling of O-linked glycosylation is best illustrated with reference to Formula

2:



Formula 2 describes a glycan structure comprising a GalNAc which is covalently linked preferably to a serine or threonine residue on a peptide backbone. While this structure is used to illustrate the most common forms of O-linked glycans, it should not be construed to limit the invention solely to these O-linked glycans. Other forms of O-linked glycans are illustrated in Figure 12. Preferred expression systems useful in the present invention express and secrete exogenous peptides having O-linked glycans comprising the GalNAc residue. Using the remodeling methods of the invention, the glycan structures on these peptides can be conveniently remodeled to generate any desired glycan structure. One of skill in the art will appreciate that O-linked glycans can be remodeled using the same principles, enzymes and reaction conditions as those available in the art once armed with the present disclosure. Exemplary reaction conditions are found throughout the Examples.

In preferred embodiments, the glycan structures are remodeled so that the structure described in Formula 2 has specific moieties. The structure of the glycan may be chosen to enhance the biological activity of the peptide, confer upon the peptide a new biological activity, remove or alter a biological activity of peptide, or better approximate the glycosylation pattern of the native peptide, among others.

In the first preferred embodiment, the peptide O-linked glycans are remodeled to better approximate the glycosylation pattern of native human proteins. In this embodiment, the glycan structure described in Formula 2 is remodeled to have the following moieties:

$X^2$  is  $|-SA$ ; or  $|-SA-SA$ ;

$f$  and  $n = 0$  or  $1$ ;

$X^{10}$  is  $SA$ ;

$m = 0$ .

This embodiment is particularly advantageous for human peptides expressed in heterologous cellular expression systems. By remodeling the O-linked glycan structures to have this configuration, the peptide can be rendered less immunogenic in a human patient and/or more stable.

In the another preferred embodiment, the peptide O-linked glycans are remodeled to display a sialylated Lewis X antigen. In this embodiment, the glycan structure described in Formula 2 is remodeled to have the following moieties:

$X^2$  is  $|-SA$ ;

5  $X^{10}$  is Fuc or  $|-GlcNAc(Fuc)-Gal-SA$ ;

f and n = 1;

m = 0.

This embodiment is particularly advantageous when the peptide which is being remodeled is most effective when targeted to a selectin molecule and cells exhibiting the same.

10 In a yet another preferred embodiment, the peptide O-linked glycans are remodeled to contain a conjugated moiety. The conjugated moiety may be a PEG molecule, another peptide, a small molecule such as a drug, among others. In this embodiment, the glycan structure described in Formula 2 is remodeled to have the following moieties:

$X^2$  is  $|-SA-R$ ;

15 f = 1;

n and m = 0;

where R is the conjugate group.

This embodiment is useful for conjugating the peptide to PEG molecules that will slow the clearance of the peptide from the patient's bloodstream, to peptides that will target both  
20 peptides to a specific tissue or cell or to another peptide of complementary therapeutic use.

It will be clear to one of skill in the art that the invention is not limited to the preferred glycan molecules described above. The preferred embodiments are only a few of the many useful glycan molecules that can be made using the remodeling methods of the invention. Those skilled in the art will know how to design other useful glycans once armed with the  
25 present invention.

In the first exemplary embodiment, the peptide is expressed in a CHO (Chinese hamster cell line) according to methods well known in the art. When a peptide with O-linked glycan consensus sites is expressed and secreted from CHO cells, the majority of the O-linked glycans will often have the structure, in the terms of Formula 2,

30  $X^2 = |-SA$ ;

f = 1;

m and n = 0.

Therefore, most of the glycans in CHO cells do not require remodeling in order to be acceptable for use in a human patient. In an exemplary embodiment, the O-linked glycans of a peptide expressed and secreted from a CHO cell are remodeled to contain a sialylated Lewis X structure by contacting the glycans with a glycosyltransferase specific for the GalNAc acceptor moiety and the fucose donor moiety in the presence of nucleotide-fucose. This process is illustrated on N-linked glycans in Figure 11 and Example 39.

In other exemplary embodiments, the peptide is expressed in insect cells such as sf9 according to methods well known in the art. When a peptide having O-linked glycan consensus sites is expressed and secreted from most sf9 cells, the majority of the O-linked glycans have the structure, in the terms of Formula 2:

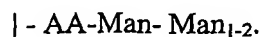
$$X^2 = H;$$

$$f = 0 \text{ or } 1;$$

$$n \text{ and } m = 0.$$

See, for example, Marchal et al., (2001, Biol. Chem. 382:151-159). In one exemplary embodiment, the O-linked glycan on a peptide expressed in an insect cell is remodeled to a humanized glycan by contacting the glycans with a glycosyltransferase specific for a GalNAc acceptor molecule and a galactose donor molecule in the presence of nucleotide-Gal; and then contacting the glycans with a glycosyltransferase specific for a Gal acceptor molecule and a SA donor molecule in the presence of nucleotide-SA. In another exemplary embodiment, the O-linked glycans are remodeled further from the humanized form to the sialylated Lewis X form by further contacting the glycans with a glycosyltransferase specific for a GalNAc acceptor molecule and a fucose donor molecule in the presence of nucleotide-fucose.

In yet another exemplary embodiment, the peptide is expressed in fungal cells, in particular *S. cerevisiae* cells, according to methods well known in the art. When a peptide with O-linked glycans consensus sites is expressed and secreted from *S. cerevisiae* cells, the majority of the O-linked glycans have the structure:



See Gemmill and Trimble (1999, Biochim. Biophys. Acta 1426:227-237). In order to remodel these O-linked glycans for use in human, it is preferable that the glycan be cleaved at the amino acid level and rebuilt from there.

In an exemplary embodiment, the glycan is the O-linked glycan on a peptide  
5 expressed in a fungal cell and is remodeled to a humanized glycan by contacting the glycan with an endoglycosylase specific for an amino acid - GalNAc bond; and then contacting the glycan with a glycosyltransferase specific for a O-linked consensus site and a GalNAc donor molecule in the presence of nucleotide-GalNAc; contacting the glycan with a  
glycosyltransferase specific for a GalNAc acceptor molecule and a galactose donor molecule  
10 in the presence of nucleotide-Gal; and then contacting the glycans with a glycosyltransferase specific for a Gal acceptor molecule and a SA donor molecule in the presence of nucleotide-SA.

Alternately, in another exemplary embodiment, the glycan is the O-linked glycan on a peptide expressed in a fungal cell and is remodeled to a humanized glycan by contacting the  
15 glycan with an protein O-mannose  $\beta$ -1,2-N-acetylglucosaminyltransferase (POMGnTI) in the presence of GlcNAc-nucleotide; then contacting the glycan with an galactosyltransferase in the presence of nucleotide-Gal; and then contracting the glycan with an sialyltransferase in the presence of nucleotide-SA.

In another exemplary embodiment, the peptide is expressed in bacterial cells, in  
20 particular *E. coli* cells, according to methods well known in the art. When a peptide with an O-linked glycan consensus site is expressed in *E. coli* cells, the O-linked consensus site will not be glycosylated. In this case, the desired glycan molecule must be built out from the peptide backbone in a manner similar to that describe for *S. cerevisiae* expression above. Further, when a peptide having an O-linked glycan is expressed in a eukaryotic cell without  
25 the proper leader sequences to direct the nascent peptide to the golgi apparatus, the mature peptide is likely not to be glycosylated. In this case as well, an O-linked glycosyl structure may be added to the peptide by building out the glycan directly from the peptide O-linked consensus site. Further, when a protein is chemically modified with a sugar moiety, it can also be remodeled as described herein.

30 These examples are meant to illustrate the invention, and not to limit it in any way. One of skill in the art will appreciate that the steps taken in each example may in some

circumstances be performed in a different order to achieve the same result. One of skill in the art will also understand that a different set of steps may also produce the same resulting glycan. Further, the preferred remodeled glycan is by no means specific to the expression system that the peptide is expressed in. The remodeled glycans are only illustrative and one of skill in the art will know how to take the principles from these examples and apply them to peptides produced in different expression systems to generate glycans not specifically described herein.

### C. Glycoconjugation, in general

The invention provides methods of preparing a conjugate of a glycosylated or an unglycosylated peptide. The conjugates of the invention are formed between peptides and diverse species such as water-soluble polymers, therapeutic moieties, diagnostic moieties, targeting moieties and the like. Also provided are conjugates that include two or more peptides linked together through a linker arm, i.e., multifunctional conjugates. The multifunctional conjugates of the invention can include two or more copies of the same peptide or a collection of diverse peptides with different structures, and/or properties.

The conjugates of the invention are formed by the enzymatic attachment of a modified sugar to the glycosylated or unglycosylated peptide. The modified sugar, when interposed between the peptide and the modifying group on the sugar becomes what is referred to herein as "an intact glycosyl linking group." Using the exquisite selectivity of enzymes, such as glycosyltransferases, the present method provides peptides that bear a desired group at one or more specific locations. Thus, according to the present invention, a modified sugar is attached directly to a selected locus on the peptide chain or, alternatively, the modified sugar is appended onto a carbohydrate moiety of a peptide. Peptides in which modified sugars are linked to both a peptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

In contrast to known chemical and enzymatic peptide elaboration strategies, the methods of the invention make it possible to assemble peptides and glycopeptides that have a substantially homogeneous derivatization pattern; the enzymes used in the invention are generally selective for a particular amino acid residue or combination of amino acid residues of the peptide or particular glycan structure. The methods are also practical for large-scale

production of modified peptides and glycopeptides. Thus, the methods of the invention provide a practical means for large-scale preparation of peptides having preselected substantially uniform derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, peptides that are

5 incompletely glycosylated during production in cell culture cells (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

The methods of the invention also provide conjugates of glycosylated and unglycosylated peptides with increased therapeutic half-life due to, for example, reduced

10 clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents can also be used to target a peptide to a particular tissue or cell surface receptor that is specific for the particular targeting agent. Moreover,

15 there is provided a class of peptides that are specifically modified with a therapeutic moiety.

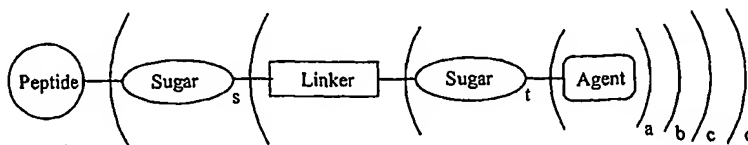
### 1. The Conjugates

In a first aspect, the present invention provides a conjugate between a peptide and a selected moiety. The link between the peptide and the selected moiety includes an intact

20 glycosyl linking group interposed between the peptide and the selected moiety. As discussed herein, the selected moiety is essentially any species that can be attached to a saccharide unit, resulting in a "modified sugar" that is recognized by an appropriate transferase enzyme, which appends the modified sugar onto the peptide. The saccharide component of the modified sugar, when interposed between the peptide and a selected moiety, becomes an

25 "intact glycosyl linking group." The glycosyl linking group is formed from any mono- or oligo-saccharide that, after modification with a selected moiety, is a substrate for an appropriate transferase.

The conjugates of the invention will typically correspond to the general structure:



in which the symbols a, b, c, d and s represent a positive, non-zero integer; and t is either 0 or a positive integer. The "agent" is a therapeutic agent, a bioactive agent, a detectable label, water-soluble moiety or the like. The "agent" can be a peptide, e.g., enzyme, antibody, antigen, etc. The linker can be any of a wide array of linking groups,  
5 *infra*. Alternatively, the linker may be a single bond or a "zero order linker." The identity of the peptide is without limitation. Exemplary peptides are provided in Figure 28.

In an exemplary embodiment, the selected moiety is a water-soluble polymer. The water-soluble polymer is covalently attached to the peptide via an intact glycosyl linking group. The glycosyl linking group is covalently attached to either an amino acid residue or a  
10 glycosyl residue of the peptide. Alternatively, the glycosyl linking group is attached to one or more glycosyl units of a glycopeptide. The invention also provides conjugates in which the glycosyl linking group is attached to both an amino acid residue and a glycosyl residue.

In addition to providing conjugates that are formed through an enzymatically added intact glycosyl linking group, the present invention provides conjugates that are highly  
15 homogenous in their substitution patterns. Using the methods of the invention, it is possible to form peptide conjugates in which essentially all of the modified sugar moieties across a population of conjugates of the invention are attached to multiple copies of a structurally identical amino acid or glycosyl residue. Thus, in a second aspect, the invention provides a peptide conjugate having a population of water-soluble polymer moieties, which are  
20 covalently linked to the peptide through an intact glycosyl linking group. In a preferred conjugate of the invention, essentially each member of the population is linked via the glycosyl linking group to a glycosyl residue of the peptide, and each glycosyl residue of the peptide to which the glycosyl linking group is attached has the same structure.

Also provided is a peptide conjugate having a population of water-soluble polymer  
25 moieties covalently linked thereto through an intact glycosyl linking group. In a preferred embodiment, essentially every member of the population of water soluble polymer moieties is linked to an amino acid residue of the peptide via an intact glycosyl linking group, and each amino acid residue having an intact glycosyl linking group attached thereto has the same structure.

30 The present invention also provides conjugates analogous to those described above in which the peptide is conjugated to a therapeutic moiety, diagnostic moiety, targeting moiety,



toxin moiety or the like via an intact glycosyl linking group. Each of the above-recited moieties can be a small molecule, natural polymer (e.g., peptide) or synthetic polymer.

In an exemplary embodiment, interleukin-2 (IL-2) is conjugated to transferrin via a bifunctional linker that includes an intact glycosyl linking group at each terminus of the PEG moiety (Scheme 1). For example, one terminus of the PEG linker is functionalized with an intact sialic acid linker that is attached to transferrin and the other is functionalized with an intact GalNAc linker that is attached to IL-2.

In another exemplary embodiment, EPO is conjugated to transferrin. In another exemplary embodiment, EPO is conjugated to glial derived neurotropic growth factor (GDNF). In these embodiments, each conjugation is accomplished via a bifunctional linker that includes an intact glycosyl linking group at each terminus of the PEG moiety, as aforementioned. Transferrin transfers the protein across the blood brain barrier.

As set forth in the Figures appended hereto, the conjugates of the invention can include intact glycosyl linking groups that are mono- or multi-valent (e.g., antennary structures), see, Figures 14-22. The conjugates of the invention also include glycosyl linking groups that are O-linked glycans originating from serine or threonine (Figure 11). Thus, conjugates of the invention include both species in which a selected moiety is attached to a peptide via a monovalent glycosyl linking group. Also included within the invention are conjugates in which more than one selected moiety is attached to a peptide via a multivalent linking group. One or more proteins can be conjugated together to take advantage of their biophysical and biological properties.

In a still further embodiment, the invention provides conjugates that localize selectively in a particular tissue due to the presence of a targeting agent as a component of the conjugate. In an exemplary embodiment, the targeting agent is a protein. Exemplary proteins include transferrin (brain, blood pool), human serum (HS)-glycoprotein (bone, brain, blood pool), antibodies (brain, tissue with antibody-specific antigen, blood pool), coagulation Factors V-XII (damaged tissue, clots, cancer, blood pool), serum proteins, e.g.,  $\alpha$ -acid glycoprotein, fetuin,  $\alpha$ -fetal protein (brain, blood pool),  $\beta$ 2-glycoprotein (liver, atherosclerosis plaques, brain, blood pool), G-CSF, GM-CSF, M-CSF, and EPO (immune stimulation, cancers, blood pool, red blood cell overproduction, neuroprotection), and albumin (increase in half-life).

In addition to the conjugates discussed above, the present invention provides methods for preparing these and other conjugates. Thus, in a further aspect, the invention provides a method of forming a covalent conjugate between a selected moiety and a peptide.

Additionally, the invention provides methods for targeting conjugates of the invention to a particular tissue or region of the body.

In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group, which is interposed between, and covalently linked to both the peptide and the modifying group (e.g., water-soluble polymer). The method includes contacting the peptide with a mixture containing a modified sugar and a glycosyltransferase for which the modified sugar is a substrate. The reaction is conducted under conditions sufficient to form a covalent bond between the modified sugar and the peptide. The sugar moiety of the modified sugar is preferably selected from nucleotide sugars, activated sugars and sugars, which are neither nucleotides nor activated.

In one embodiment, the invention provides a method for linking two or more peptides through a linking group. The linking group is of any useful structure and may be selected from straight-chain and branched chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified sugar (i.e., a nascent intact glycosyl linking group).

In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a PEG linker. The construct conforms to the general structure set forth in the cartoon above. As described herein, the construct of the invention includes two intact glycosyl linking groups (i.e.,  $s + t = 1$ ). The focus on a PEG linker that includes two glycosyl groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention.

Thus, a PEG moiety is functionalized at a first terminus with a first glycosyl unit and at a second terminus with a second glycosyl unit. The first and second glycosyl units are preferably substrates for different transferases, allowing orthogonal attachment of the first and second peptides to the first and second glycosyl units, respectively. In practice, the (glycosyl)<sup>1</sup>-PEG-(glycosyl)<sup>2</sup> linker is contacted with the first peptide and a first transferase

for which the first glycosyl unit is a substrate, thereby forming (peptide)<sup>1</sup>-(glycosyl)<sup>1</sup>-PEG-(glycosyl)<sup>2</sup>. The first transferase and/or unreacted peptide is then optionally removed from the reaction mixture. The second peptide and a second transferase for which the second glycosyl unit is a substrate are added to the

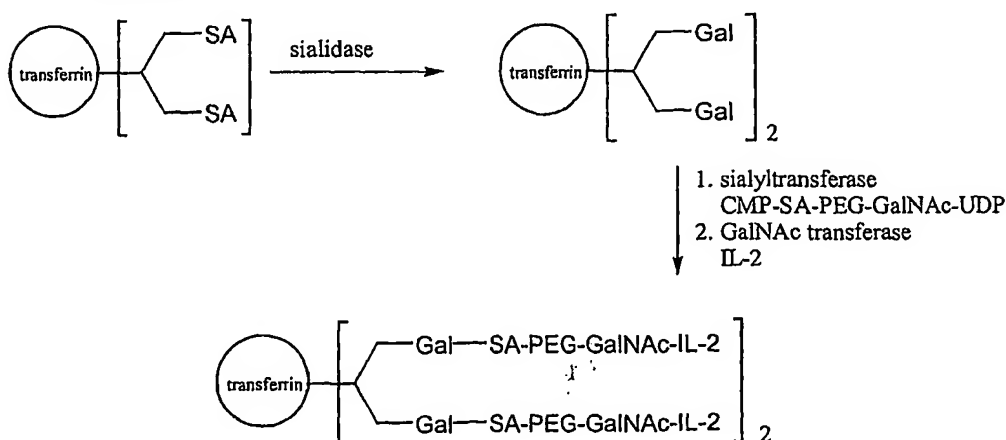
5 (peptide)<sup>1</sup>-(glycosyl)<sup>1</sup>-PEG-(glycosyl)<sup>2</sup> conjugate, forming (peptide)<sup>1</sup>-(glycosyl)<sup>1</sup>-PEG-(glycosyl)<sup>2</sup>-(peptide)<sup>2</sup>. Those of skill in the art will appreciate that the method outlined above is also applicable to forming conjugates between more than two peptides by, for example, the use of a branched PEG, dendrimer, poly(amino acid), polysaccharide or the like.

10 As noted previously, in an exemplary embodiment, interleukin-2 (IL-2) is conjugated to transferrin via a bifunctional linker that includes an intact glycosyl linking group at each terminus of the PEG moiety (Scheme 1). The IL-2 conjugate has an *in vivo* half-life that is increased over that of IL-2 alone by virtue of the greater molecular size of the conjugate. Moreover, the conjugation of IL-2 to transferrin serves to selectively target the conjugate to

15 the brain. For example, one terminus of the PEG linker is functionalized with a CMP-sialic acid and the other is functionalized with an UDP-GalNAc. The linker is combined with IL-2 in the presence of a GalNAc transferase, resulting in the attachment of the GalNAc of the linker arm to a serine and/or threonine residue on the IL-2.

20 In another exemplary embodiment, transferrin is conjugated to a nucleic acid for use in gene therapy.

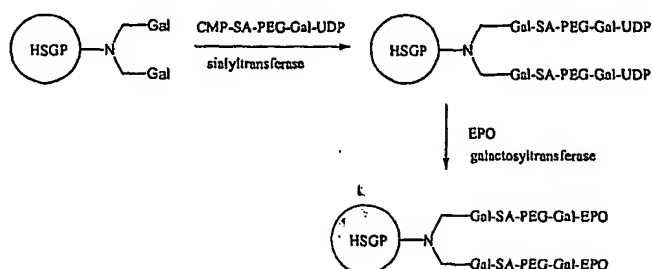
**Scheme 1**



The processes described above can be carried through as many cycles as desired, and is not limited to forming a conjugate between two peptides with a single linker. Moreover, those of skill in the art will appreciate that the reactions functionalizing the intact glycosyl linking groups at the termini of the PEG (or other) linker with the peptide can occur simultaneously in the same reaction vessel, or they can be carried out in a step-wise fashion. When the reactions are carried out in a step-wise manner, the conjugate produced at each step is optionally purified from one or more reaction components (e.g., enzymes, peptides).

A still further exemplary embodiment is set forth in Scheme 2. Scheme 2 shows a method of preparing a conjugate that targets a selected protein, e.g., EPO, to bone and increases the circulatory half-life of the selected protein.

Scheme 2

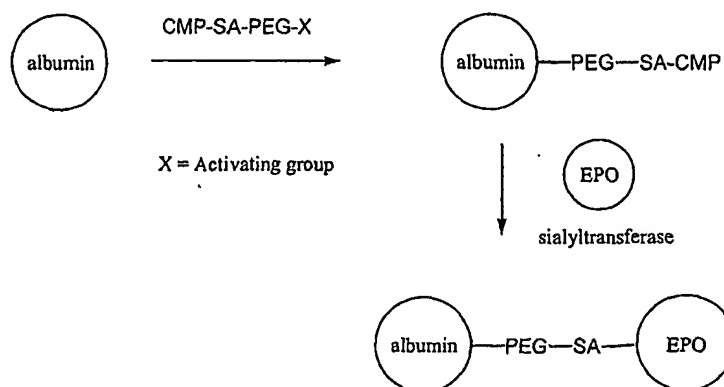


The use of reactive derivatives of PEG (or other linkers) to attach one or more peptide moieties to the linker is within the scope of the present invention. The invention is not limited by the identity of the reactive PEG analogue. Many activated derivatives of poly(ethylene glycol) are available commercially and in the literature. It is well within the abilities of one of skill to choose, and synthesize if necessary, an appropriate activated PEG derivative with which to prepare a substrate useful in the present invention. See, Abuchowski *et al. Cancer Biochem. Biophys.*, **7**: 175-186 (1984); Abuchowski *et al., J. Biol. Chem.*, **252**: 3582-3586 (1977); Jackson *et al., Anal. Biochem.*, **165**: 114-127 (1987); Koide *et al., Biochem Biophys. Res. Commun.*, **111**: 659-667 (1983)), tresylate (Nilsson *et al., Methods Enzymol.*, **104**: 56-69 (1984); Delgado *et al., Biotechnol. Appl. Biochem.*, **12**: 119-128 (1990)); N-hydroxysuccinimide derived active esters (Buckmann *et al., Makromol. Chem.*, **182**: 1379-1384 (1981); Joppich *et al., Makromol. Chem.*, **180**: 1381-1384 (1979); Abuchowski *et al., Cancer Biochem. Biophys.*, **7**: 175-186 (1984); Katreet *al. Proc. Natl. Acad. Sci. U.S.A.*, **84**: 1487-1491 (1987); Kitamura *et al., Cancer Res.*, **51**: 4310-4315

(1991); Boccu *et al.*, *Z. Naturforsch.*, **38C**: 94-99 (1983), carbonates (Zalipsky *et al.*, POLY(ETHYLENE GLYCOL) CHEMISTRY: BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, Harris, Ed., Plenum Press, New York, 1992, pp. 347-370; Zalipsky *et al.*, *Biotechnol. Appl. Biochem.*, **15**: 100-114 (1992); Veronese *et al.*, *Appl. Biochem. Biotech.*, **11**: 141-152 (1985)), imidazolyl formates (Beauchamp *et al.*, *Anal. Biochem.*, **131**: 25-33 (1983); Berger *et al.*, *Blood*, **71**: 1641-1647 (1988)), 4-dithiopyridines (Woghiren *et al.*, *Bioconjugate Chem.*, **4**: 314-318 (1993)), isocyanates (Byun *et al.*, *ASAIO Journal*, M649-M-653 (1992)) and epoxides (U.S. Pat. No. 4,806,595, issued to Noishiki *et al.*, (1989). Other linking groups include the urethane linkage between amino groups and activated PEG. See, Veronese, *et al.*, *Appl. Biochem. Biotechnol.*, **11**: 141-152 (1985).

In another exemplary embodiment in which a reactive PEG derivative is utilized, the invention provides a method for extending the blood-circulation half-life of a selected peptide, in essence targeting the peptide to the blood pool, by conjugating the peptide to a synthetic or natural polymer of a size sufficient to retard the filtration of the protein by the glomerulus (e.g., albumin). This embodiment of the invention is illustrated in Scheme 3 in which erythropoietin (EPO) is conjugated to albumin via a PEG linker using a combination of chemical and enzymatic modification.

Scheme 3



Thus, as shown in Scheme 3, an amino acid residue of albumin is modified with a reactive PEG derivative, such as X-PEG-(CMP-sialic acid), in which X is an activating group (e.g., active ester, isothiocyanate, etc). The PEG derivative and EPO are combined and contacted with a transferase for which CMP-sialic acid is a substrate. In a further illustrative embodiment, an  $\epsilon$ -amine of lysine is reacted with the N-hydroxysuccinimide ester of the

PEG-linker to form the albumin conjugate. The CMP-sialic acid of the linker is enzymatically conjugated to an appropriate residue on EPO, e.g., Gal, thereby forming the conjugate. Those of skill will appreciate that the above-described method is not limited to the reaction partners set forth. Moreover, the method can be practiced to form conjugates that include more than two protein moieties by, for example, utilizing a branched linker having more than two termini.

## 2. Modified Sugars

Modified glycosyl donor species ("modified sugars") are preferably selected from modified sugar nucleotides, activated modified sugars and modified sugars that are simple saccharides that are neither nucleotides nor activated. Any desired carbohydrate structure can be added to a peptide using the methods of the invention. Typically, the structure will be a monosaccharide, but the present invention is not limited to the use of modified monosaccharide sugars; oligosaccharides and polysaccharides are useful as well.

The modifying group is attached to a sugar moiety by enzymatic means, chemical means or a combination thereof, thereby producing a modified sugar. The sugars are substituted at any position that allows for the attachment of the modifying moiety, yet which still allows the sugar to function as a substrate for the enzyme used to ligate the modified sugar to the peptide. In a preferred embodiment, when sialic acid is the sugar, the sialic acid is substituted with the modifying group at either the 9-position on the pyruvyl side chain or at the 5-position on the amine moiety that is normally acetylated in sialic acid.

In certain embodiments of the present invention, a modified sugar nucleotide is utilized to add the modified sugar to the peptide. Exemplary sugar nucleotides that are used in the present invention in their modified form include nucleotide mono-, di- or triphosphates or analogs thereof. In a preferred embodiment, the modified sugar nucleotide is selected from a UDP-glycoside, CMP-glycoside, or a GDP-glycoside. Even more preferably, the modified sugar nucleotide is selected from an UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, GDP-mannose, GDP-fucose, CMP-sialic acid, or CMP-NeuAc. N-acetylamines derivatives of the sugar nucleotides are also of use in the method of the invention.

The invention also provides methods for synthesizing a modified peptide using a modified sugar, *e.g.*, modified-galactose, -fucose, and -sialic acid. When a modified sialic acid is used, either a sialyltransferase or a trans-sialidase (for  $\alpha$ 2,3-linked sialic acid only) can be used in these methods.

5 In other embodiments, the modified sugar is an activated sugar. Activated modified sugars, which are useful in the present invention are typically glycosides which have been synthetically altered to include an activated leaving group. As used herein, the term "activated leaving group" refers to those moieties, which are easily displaced in enzyme-regulated nucleophilic substitution reactions. Many activated sugars are known in the art.

10 See, for example, Vocadlo et al., In CARBOHYDRATE CHEMISTRY AND BIOLOGY, Vol. 2, Ernst et al. Ed., Wiley-VCH Verlag: Weinheim, Germany, 2000; Kodama et al., *Tetrahedron Lett.* 34: 6419 (1993); Loughheed, et al., *J. Biol. Chem.* 274: 37717 (1999)).

Examples of activating groups (leaving groups) include fluoro, chloro, bromo, tosylate ester, mesylate ester, triflate ester and the like. Preferred activated leaving groups,  
15 for use in the present invention, are those that do not significantly sterically encumber the enzymatic transfer of the glycoside to the acceptor. Accordingly, preferred embodiments of activated glycoside derivatives include glycosyl fluorides and glycosyl mesylates, with glycosyl fluorides being particularly preferred. Among the glycosyl fluorides,  $\alpha$ -galactosyl fluoride,  $\alpha$ -mannosyl fluoride,  $\alpha$ -glucosyl fluoride,  $\alpha$ -fucosyl fluoride,  $\alpha$ -xylosyl fluoride,  $\alpha$ -  
20 sialyl fluoride,  $\alpha$ -N-acetylglucosaminyl fluoride,  $\alpha$ -N-acetylgalactosaminyl fluoride,  $\beta$ -galactosyl fluoride,  $\beta$ -mannosyl fluoride,  $\beta$ -glucosyl fluoride,  $\beta$ -fucosyl fluoride,  $\beta$ -xylosyl fluoride,  $\beta$ -sialyl fluoride,  $\beta$ -N-acetylglucosaminyl fluoride and  $\beta$ -N-acetylgalactosaminyl fluoride are most preferred.

By way of illustration, glycosyl fluorides can be prepared from the free sugar by first  
25 acetylating the sugar and then treating it with HF/pyridine. This generates the thermodynamically most stable anomer of the protected (acetylated) glycosyl fluoride (*i.e.*, the  $\alpha$ -glycosyl fluoride). If the less stable anomer (*i.e.*, the  $\beta$ -glycosyl fluoride) is desired, it can be prepared by converting the peracetylated sugar with HBr/HOAc or with HCl to generate the anomeric bromide or chloride. This intermediate is reacted with a fluoride salt  
30 such as silver fluoride to generate the glycosyl fluoride. Acetylated glycosyl fluorides may

be deprotected by reaction with mild (catalytic) base in methanol (*e.g.* NaOMe/MeOH). In addition, many glycosyl fluorides are commercially available.

Other activated glycosyl derivatives can be prepared using conventional methods known to those of skill in the art. For example, glycosyl mesylates can be prepared by treatment of the fully benzylated hemiacetal form of the sugar with mesyl chloride, followed by catalytic hydrogenation to remove the benzyl groups.

In a further exemplary embodiment, the modified sugar is an oligosaccharide having an antennary structure. In a preferred embodiment, one or more of the termini of the antennae bear the modifying moiety. When more than one modifying moiety is attached to an oligosaccharide having an antennary structure, the oligosaccharide is useful to "amplify" the modifying moiety; each oligosaccharide unit conjugated to the peptide attaches multiple copies of the modifying group to the peptide. The general structure of a typical chelate of the invention as set forth in the drawing above, encompasses multivalent species resulting from preparing a conjugate of the invention utilizing an antennary structure. Many antennary saccharide structures are known in the art, and the present method can be practiced with them without limitation.

Exemplary modifying groups are discussed below. The modifying groups can be selected for one or more desirable property. Exemplary properties include, but are not limited to, enhanced pharmacokinetics, enhanced pharmacodynamics, improved biodistribution, providing a polyvalent species, improved water solubility, enhanced or diminished lipophilicity, and tissue targeting.

#### D. Peptide Conjugates

##### a) Water-Soluble Polymers

The hydrophilicity of a selected peptide is enhanced by conjugation with polar molecules such as amine-, ester-, hydroxyl- and polyhydroxyl-containing molecules. Representative examples include, but are not limited to, polylysine, polyethyleneimine, poly(ethylene glycol) and poly(propyleneglycol). Preferred water-soluble polymers are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Polymers that are not naturally occurring sugars may be used. In addition, the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, poly(ethylene glycol),



poly(propylene glycol), poly(aspartate), biomolecule, therapeutic moiety, diagnostic moiety, *etc.*) is also contemplated. In another exemplary embodiment, a therapeutic sugar moiety is conjugated to a linker arm and the sugar-linker arm is subsequently conjugated to a peptide via a method of the invention.

5       Methods and chemistry for activation of water-soluble polymers and saccharides as well as methods for conjugating saccharides and polymers to various species are described in the literature. Commonly used methods for activation of polymers include activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxydes, epichlorohydrin, divinylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine, *etc.* (*see*, R. F. Taylor, (1991), PROTEIN IMMOBILISATION. FUNDAMENTALS AND APPLICATIONS, Marcel Dekker, N.Y.; S. S. Wong, (1992), CHEMISTRY OF PROTEIN CONJUGATION AND  
10       CROSSLINKING, CRC Press, Boca Raton; G. T. Hermanson *et al.*, (1993), IMMOBILIZED AFFINITY LIGAND TECHNIQUES, Academic Press, N.Y.; Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American  
15       Chemical Society, Washington, D.C. 1991).

      Routes for preparing reactive PEG molecules and forming conjugates using the reactive molecules are known in the art. For example, U.S. Patent No. 5,672,662 discloses a water soluble and isolatable conjugate of an active ester of a polymer acid selected from linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols),  
20       and poly(acrylomorpholine), wherein the polymer has about 44 or more recurring units.

      U.S. Patent No. 6,376,604 sets forth a method for preparing a water-soluble 1-benzotriazolylcarbonate ester of a water-soluble and non-peptidic polymer by reacting a terminal hydroxyl of the polymer with di(1-benzotriazolyl)carbonate in an organic solvent. The active ester is used to form conjugates with a biologically active agent such as a protein  
25       or peptide.

      WO 99/45964 describes a conjugate comprising a biologically active agent and an activated water soluble polymer comprising a polymer backbone having at least one terminus linked to the polymer backbone through a stable linkage, wherein at least one terminus comprises a branching moiety having proximal reactive groups linked to the branching  
30       moiety, in which the biologically active agent is linked to at least one of the proximal reactive groups. Other branched poly(ethylene glycols) are described in WO 96/21469, U.S. Patent

No. 5,932,462 describes a conjugate formed with a branched PEG molecule that includes a branched terminus that includes reactive functional groups. The free reactive groups are available to react with a biologically active species, such as a protein or peptide, forming conjugates between the poly(ethylene glycol) and the biologically active species. U.S. Patent  
5 No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a peptide at each of the PEG linker termini.

Conjugates that include degradable PEG linkages are described in WO 99/34833; and WO 99/14259, as well as in U.S. Patent No. 6,348,558. Such degradable linkages are applicable in the present invention.

10 Although both reactive PEG derivatives and conjugates formed using the derivatives are known in the art, until the present invention, it was not recognized that a conjugate could be formed between PEG (or other polymer) and another species, such as a peptide or glycopeptide, through an intact glycosyl linking group.

Many water-soluble polymers are known to those of skill in the art and are useful in  
15 practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (e.g., dextran, amylose, hyaluronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids), e.g., poly(glutamic acid); nucleic acids; synthetic polymers (e.g., poly(acrylic acid), poly(ethers), e.g., poly(ethylene glycol); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole  
20 limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

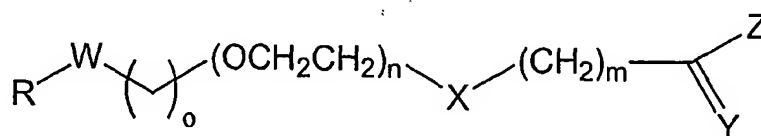
Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation  
25 between activated polymers and peptides, e.g. Coagulation Factor VIII (WO 94/15625), hemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese *et al.*, *App. Biochem. Biotech.* 11: 141-45 (1985)).

Preferred water-soluble polymers are those in which a substantial proportion of the  
30 polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are "homodisperse."

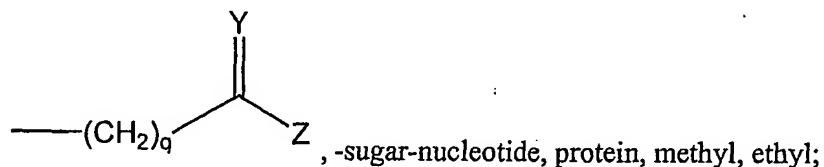
The present invention is further illustrated by reference to a poly(ethylene glycol) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG are available. See, for example, Harris, *Macromol. Chem. Phys.* **C25**: 325-373 (1985); Scouten, *Methods in Enzymology* **135**: 30-65 (1987); Wong *et al.*, *Enzyme Microb. Technol.* **14**: 866-874 (1992); Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995); and Bhadra, *et al.*, *Pharmazie*, **57**:5-29 (2002).

Poly(ethylene glycol) molecules suitable for use in the invention include, but are not limited to, those described by the following Formula 3:

10      **Formula 3.**



R= H, alkyl, benzyl, aryl, acetal, OHC-, H<sub>2</sub>N-CH<sub>2</sub>CH<sub>2</sub>-, HS-CH<sub>2</sub>CH<sub>2</sub>-,



15      X, Y, W, U (independently selected) = O, S, NH, N-R';

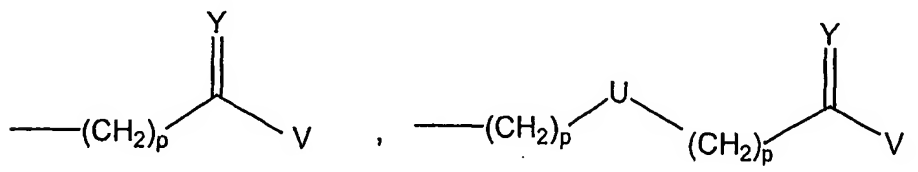
R', R''' (independently selected) = alkyl, benzyl, aryl, alkyl aryl, pyridyl, substituted aryl, arylalkyl, acylaryl;

n = 1 to 2000;

m, q, p (independently selected) = 0 to 20

20      o = 0 to 20;

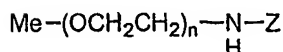
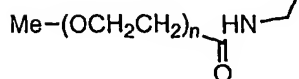
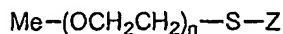
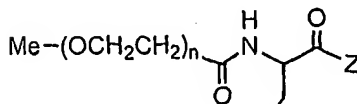
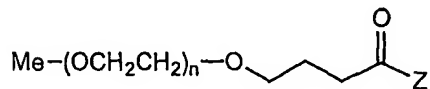
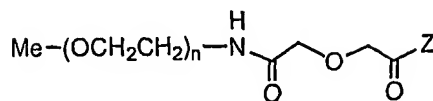
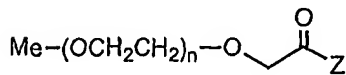
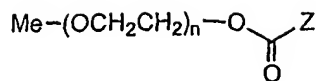
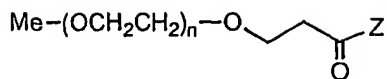
Z = HO, NH<sub>2</sub>, halogen, S-R''', activated esters,



-sugar-nucleotide, protein, imidazole, HOBT, tetrazole, halide; and

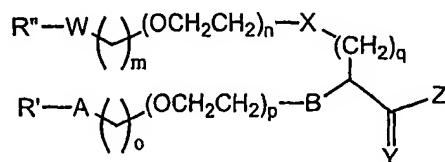
V = HO, NH<sub>2</sub>, halogen, S-R''', activated esters, activated amides; -sugar-nucleotide, protein.

In preferred embodiments, the poly(ethylene glycol) molecule is selected from the following:



The poly(ethylene glycol) useful in forming the conjugate of the invention is either linear or branched. Branched poly(ethylene glycol) molecules suitable for use in the invention include, but are not limited to, those described by the following Formula:

**Formula 4:**



5

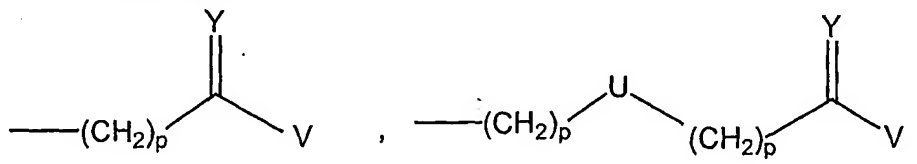
R', R'', R''' (independently selected) = H, alkyl, benzyl, aryl, acetal, OHC-, H<sub>2</sub>N-CH<sub>2</sub>CH<sub>2</sub>-, HS-CH<sup>2</sup>CH<sub>2</sub>-, -(CH<sub>2</sub>)<sub>q</sub>CY-Z, -sugar-nucleotide, protein, methyl, ethyl, heteroaryl, acylalkyl, acylaryl, acylalkylaryl;

X, Y, W, A, B (independently selected) = O, S, NH, N-R', (CH<sub>2</sub>)<sub>i</sub>;

10 n, p (independently selected) = 1 to 2000;

m, q, o (independently selected) = 0 to 20;

Z = HO, NH<sub>2</sub>, halogen, S-R''', activated esters,



-sugar-nucleotide, protein;

15 V = HO, NH<sub>2</sub>, halogen, S-R''', activated esters, activated amides,

-sugar-nucleotide, protein.

The *in vivo* half-life, area under the curve, and/or residence time of therapeutic peptides can also be enhanced with water-soluble polymers such as polyethylene glycol (PEG) and polypropylene glycol (PPG). For example, chemical modification of proteins with PEG (PEGylation) increases their molecular size and decreases their surface- and functional group-accessibility, each of which are dependent on the size of the PEG attached to the protein. This results in an improvement of plasma half-lives and in proteolytic-stability, and a decrease in immunogenicity and hepatic uptake (Chaffee *et al. J. Clin. Invest.* 89: 1643-1651 (1992); Pyatak *et al. Res. Commun. Chem. Pathol Pharmacol.* 29: 113-127 (1980)). PEGylation of interleukin-2 has been reported to increase its antitumor potency *in vivo* (Katre *et al. Proc. Natl. Acad. Sci. USA.* 84: 1487-1491 (1987)) and PEGylation of a F(ab')<sub>2</sub> derived from the monoclonal antibody A7 has improved its tumor localization (Kitamura *et al. Biochem. Biophys. Res. Commun.* 28: 1387-1394 (1990)).

In one preferred embodiment, the *in vivo* half-life of a peptide derivatized with a water-soluble polymer by a method of the invention is increased relevant to the *in vivo* half-life of the non-derivatized peptide. In another preferred embodiment, the area under the curve of a peptide derivatized with a water-soluble polymer using a method of the invention is increased relevant to the area under the curve of the non-derivatized peptide. In another preferred embodiment, the residence time of a peptide derivatized with a water-soluble polymer using a method of the invention is increased relevant to the residence time of the non-derivatized peptide. Techniques to determine the *in vivo* half-life, the area under the curve and the residence time are well known in the art. Descriptions of such techniques can be found in J.G. Wagner, 1993, Pharmacokinetics for the Pharmaceutical Scientist, Technomic Publishing Company, Inc. Lancaster PA.

The increase in peptide *in vivo* half-life is best expressed as a range of percent increase in this quantity. The lower end of the range of percent increase is about 40%, about 60%, about 80%, about 100%, about 150% or about 200%. The upper end of the range is about 60%, about 80%, about 100%, about 150%, or more than about 250%.

In an exemplary embodiment, the present invention provides a PEGylated follicle stimulating hormone (Examples 23 and 24). In a further exemplary embodiment, the invention provides a PEGylated transferrin (Example 42).

Other exemplary water-soluble polymers of use in the invention include, but are not limited to linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols), and poly(acrylomorpholine), dextran, starch, poly(amino acids), etc.

b) Water-insoluble polymers

5       The conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. *See, for example, Dunn et al., Eds. POLYMERIC*  
10 *DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.* Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes,  
15 polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate),  
20 poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

Synthetically modified natural polymers of use in conjugates of the invention include,  
25 but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt,  
30 and polymers of acrylic and methacrylic esters and alginic acid.

These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton, PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

5 Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those  
10 including collagen, pluronics and the like.

The polymers of use in the invention include "hybrid" polymers that include water-insoluble materials having within at least a portion of their structure, a bioresorbable molecule. An example of such a polymer is one that includes a water-insoluble copolymer, which has a bioresorbable region, a hydrophilic region and a plurality of crosslinkable  
15 functional groups per polymer chain.

For purposes of the present invention, "water-insoluble materials" includes materials that are substantially insoluble in water or water-containing environments. Thus, although certain regions or segments of the copolymer may be hydrophilic or even water-soluble, the polymer molecule, as a whole, does not to any substantial measure dissolve in water.

20 For purposes of the present invention, the term "bioresorbable molecule" includes a region that is capable of being metabolized or broken down and resorbed and/or eliminated through normal excretory routes by the body. Such metabolites or break down products are preferably substantially non-toxic to the body.

The bioresorbable region may be either hydrophobic or hydrophilic, so long as the  
25 copolymer composition as a whole is not rendered water-soluble. Thus, the bioresorbable region is selected based on the preference that the polymer, as a whole, remains water-insoluble. Accordingly, the relative properties, *i.e.*, the kinds of functional groups contained by, and the relative proportions of the bioresorbable region, and the hydrophilic region are selected to ensure that useful bioresorbable compositions remain water-insoluble.

30 Exemplary resorbable polymers include, for example, synthetically produced resorbable block copolymers of poly( $\alpha$ -hydroxy-carboxylic acid)/poly(oxyalkylene, (*see*,



Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. *See*, Younes *et al.*, *J Biomed. Mater. Res.* 21: 1301-1316 (1987); and Cohn *et al.*, *J Biomed. Mater. Res.* 22: 993-1009 (1988).

5        Presently preferred bioresorbable polymers include one or more components selected from poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly (amino acids), poly(anhydrides), poly(orthoesters), poly(carbonates), poly(phosphazines), poly(phosphoesters), poly(thioesters), polysaccharides and mixtures thereof. More preferably still, the biosresorbable polymer includes a poly(hydroxy) acid  
10        component. Of the poly(hydroxy) acids, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid and copolymers and mixtures thereof are preferred.

      In addition to forming fragments that are absorbed *in vivo* ("bioresorbed"), preferred polymeric coatings for use in the methods of the invention can also form an excretable and/or metabolizable fragment.

15        Higher order copolymers can also be used in the present invention. For example, Casey *et al.*, U.S. Patent No. 4,438,253, which issued on March 20, 1984, discloses tri-block copolymers produced from the transesterification of poly(glycolic acid) and an hydroxyl-ended poly(alkylene glycol). Such compositions are disclosed for use as resorbable monofilament sutures. The flexibility of such compositions is controlled by the incorporation  
20        of an aromatic orthocarbonate, such as tetra-p-tolyl orthocarbonate into the copolymer structure.

      Other coatings based on lactic and/or glycolic acids can also be utilized. For example, Spinu, U.S. Patent No. 5,202,413, which issued on April 13, 1993, discloses biodegradable multi-block copolymers having sequentially ordered blocks of polylactide and/or  
25        polyglycolide produced by ring-opening polymerization of lactide and/or glycolide onto either an oligomeric diol or a diamine residue followed by chain extension with a difunctional compound, such as, a diisocyanate, diacylchloride or dichlorosilane.

      Bioresorbable regions of coatings useful in the present invention can be designed to be hydrolytically and/or enzymatically cleavable. For purposes of the present invention,  
30        "hydrolytically cleavable" refers to the susceptibility of the copolymer, especially the bioresorbable region, to hydrolysis in water or a water-containing environment. Similarly,

"enzymatically cleavable" as used herein refers to the susceptibility of the copolymer, especially the bioresorbable region, to cleavage by endogenous or exogenous enzymes.

When placed within the body, the hydrophilic region can be processed into excretable and/or metabolizable fragments. Thus, the hydrophilic region can include, for example, polyethers, polyalkylene oxides, polyols, poly(vinyl pyrrolidine), poly(vinyl alcohol), poly(alkyl oxazolines), polysaccharides, carbohydrates, peptides, proteins and copolymers and mixtures thereof. Furthermore, the hydrophilic region can also be, for example, a poly(alkylene) oxide. Such poly(alkylene) oxides can include, for example, poly(ethylene) oxide, poly(propylene) oxide and mixtures and copolymers thereof.

Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or more of these properties.

Bio-compatible hydrogel compositions whose integrity can be controlled through crosslinking are known and are presently preferred for use in the methods of the invention. For example, Hubbell *et al.*, U.S. Patent Nos. 5,410,016, which issued on April 25, 1995 and 5,529,914, which issued on June 25, 1996, disclose water-soluble systems, which are crosslinked block copolymers having a water-soluble central block segment sandwiched between two hydrolytically labile extensions. Such copolymers are further end-capped with photopolymerizable acrylate functionalities. When crosslinked, these systems become hydrogels. The water soluble central block of such copolymers can include poly(ethylene glycol); whereas, the hydrolytically labile extensions can be a poly( $\alpha$ -hydroxy acid), such as polyglycolic acid or polylactic acid. See, Sawhney *et al.*, *Macromolecules* 26: 581-587 (1993).

In another preferred embodiment, the gel is a thermoreversible gel. Thermoreversible gels including components, such as pluronics, collagen, gelatin, hyaluronic acid,

polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and combinations thereof are presently preferred.

In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those skilled in the art, for example, as described in Eppstein *et al.*, U.S. Patent No. 4,522,811, which issued on June 11, 1985. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearyl phosphatidyl ethanolamine, stearyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

The above-recited microparticles and methods of preparing the microparticles are offered by way of example and they are not intended to define the scope of microparticles of use in the present invention. It will be apparent to those of skill in the art that an array of microparticles, fabricated by different methods, are of use in the present invention.

#### c) Biomolecules

In another preferred embodiment, the modified sugar bears a biomolecule. In still further preferred embodiments, the biomolecule is a functional protein, enzyme, antigen, antibody, peptide, nucleic acid (*e.g.*, single nucleotides or nucleosides, oligonucleotides, polynucleotides and single- and higher-stranded nucleic acids), lectin, receptor or a combination thereof.

Some preferred biomolecules are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Other biomolecules may be fluorescent. The use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, PEG, biomolecule, therapeutic moiety, diagnostic moiety, *etc.*) is appropriate. In an exemplary embodiment, a sugar moiety, which is a biomolecule, is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or they can be produced by synthetic methods. Peptides can be natural peptides or mutated peptides. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Peptides useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal; either intact or fragments. The peptides are optionally the products of a program of directed evolution.

Both naturally derived and synthetic peptides and nucleic acids are of use in conjunction with the present invention; these molecules can be attached to a sugar residue component or a crosslinking agent by any available reactive group. For example, peptides can be attached through a reactive amine, carboxyl, sulfhydryl, or hydroxyl group. The reactive group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (e.g., exocyclic amine) or an available hydroxyl group on a sugar moiety (e.g., 3'- or 5'-hydroxyl). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. See, Chrissey *et al.* *Nucleic Acids Res.* **24**: 3031-3039 (1996).

In a further preferred embodiment, the biomolecule is selected to direct the peptide modified by the methods of the invention to a specific tissue, thereby enhancing the delivery of the peptide to that tissue relative to the amount of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized peptide delivered to a specific tissue within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about 100%. Presently, preferred biomolecules for targeting applications include antibodies, hormones and ligands for cell-surface receptors. Exemplary targeting biomolecules include, but are not limited to, an antibody specific for the transferrin receptor for delivery of the molecule to the brain (Penichet *et al.*, 1999, *J. Immunol.* 163:4421-4426; Pardridge, 2002, *Adv. Exp. Med. Biol.* 513:397-430), a peptide that recognizes the vasculature of the prostate (Arap *et al.*, 2002, *PNAS* 99:1527-1531), and an antibody specific for lung caveolae (McIntosh *et al.*, 2002, *PNAS* 99:1996-2001).

In a presently preferred embodiment, the modifying group is a protein. In an exemplary embodiment, the protein is an interferon. The interferons are antiviral glycoproteins that, in humans, are secreted by human primary fibroblasts after induction with virus or double-stranded RNA. Interferons are of interest as therapeutics, e.g., antivirals and treatment of multiple sclerosis. For references discussing interferon- $\beta$ , see, e.g., Yu, *et al.*, *J. Neuroimmunol.*, **64**(1):91-100 (1996); Schmidt, J., *J. Neurosci. Res.*, **65**(1):59-67 (2001); Wender, *et al.*, *Folia Neuropathol.*, **39**(2):91-93 (2001); Martin, *et al.*, *Springer Semin. Immunopathol.*, **18**(1):1-24 (1996); Takane, *et al.*, *J. Pharmacol. Exp. Ther.*, **294**(2):746-752 (2000); Sburlati, *et al.*, *Biotechnol. Prog.*, **14**:189-192 (1998); Dodd, *et al.*, *Biochimica et Biophysica Acta*, **787**:183-187 (1984); Edelbaum, *et al.*, *J. Interferon Res.*, **12**:449-453 (1992); Conradt, *et al.*, *J. Biol. Chem.*, **262**(30):14600-14605 (1987); Civas, *et al.*, *Eur. J. Biochem.*, **173**:311-316 (1988); Demolder, *et al.*, *J. Biotechnol.*, **32**:179-189 (1994); Sedmak, *et al.*, *J. Interferon Res.*, **9**(Suppl 1):S61-S65 (1989); Kagawa, *et al.*, *J. Biol. Chem.*, **263**(33):17508-17515 (1988); Hershenson, *et al.*, U.S. Patent No. 4,894,330; Jayaram, *et al.*, *J. Interferon Res.*, **3**(2):177-180 (1983); Menge, *et al.*, *Develop. Biol. Standard.*, **66**:391-401 (1987); Vonk, *et al.*, *J. Interferon Res.*, **3**(2):169-175 (1983); and Adolf, *et al.*, *J. Interferon Res.*, **10**:255-267 (1990). For references relevant to interferon- $\alpha$ , see, Asano, *et al.*, *Eur. J. Cancer*, **27**(Suppl 4):S21-S25 (1991); Nagy, *et al.*, *Anticancer Research*, **8**(3):467-470 (1988); Dron, *et al.*, *J. Biol. Regul. Homeost. Agents*, **3**(1):13-19 (1989); Habib, *et al.*, *Am. Surg.*, **67**(3):257-260 (3/2001); and Sugiyama, *et al.*, *Eur. J. Biochem.*, **217**:921-927 (1993).

In an exemplary interferon conjugate, interferon  $\beta$  is conjugated to a second peptide via a linker arm. The linker arm includes an intact glycosyl linking group through which it is attached to the second peptide via a method of the invention. The linker arm also optionally includes a second intact glycosyl linking group, through which it is attached to the interferon.

In another exemplary embodiment, the invention provides a conjugate of follicle stimulating hormone (FSH). FSH is a glycoprotein hormone. See, for example, Saneyoshi, *et al.*, *Biol. Reprod.*, **65**:1686-1690 (2001); Hakola, *et al.*, *J. Endocrinol.*, **158**:441-448 (1998); Stanton, *et al.*, *Mol. Cell. Endocrinol.*, **125**:133-141 (1996); Walton, *et al.*, *J. Clin. Endocrinol. Metab.*, **86**(8):3675-3685 (08/2001); Ulloa-Aguirre, *et al.*, *Endocrine*, **11**(3):205-215 (12/1999); Castro-Fernández, *et al.*, *J. Clin. Endocrinol. Metab.*, **85**(12):4603-4610 (2000); Prevost, Rebecca R., *Pharmacotherapy*, **18**(5):1001-1010 (1998); Linskens, *et al.*,

*The FASEB Journal*, 13:639-645 (04/1999); Butnev, *et al.*, *Biol. Reprod.*, 58:458-469 (1998); Muyan, *et al.*, *Mol. Endo.*, 12(5):766-772 (1998); Min, *et al.*, *Endo. J.*, 43(5):585-593 (1996); Boime, *et al.*, *Recent Progress in Hormone Research*, 34:271-289 (1999); and Rafferty, *et al.*, *J. Endo.*, 145:527-533 (1995). The FSH conjugate can be formed in a manner similar to that  
5 described for interferon.

In yet another exemplary embodiment, the conjugate includes erythropoietin (EPO). EPO is known to mediate response to hypoxia and to stimulate the production of red blood cells. For pertinent references, *see*, Cerami, *et al.*, *Seminars in Oncology*, 28(2)(Suppl 8):66-70 (04/2001). An exemplary EPO conjugate is formed analogously to the conjugate of  
10 interferon.

In a further exemplary embodiment, the invention provides a conjugate of human granulocyte colony stimulating factor (G-CSF). G-CSF is a glycoprotein that stimulates proliferation, differentiation and activation of neutropoietic progenitor cells into functionally mature neutrophils. Injected G-CSF is known to be rapidly cleared from the body. *See*, for  
15 example, Nohynek, *et al.*, *Cancer Chemother. Pharmacol.*, 39:259-266 (1997); Lord, *et al.*, *Clinical Cancer Research*, 7(7):2085-2090 (07/2001); Rotondaro, *et al.*, *Molecular Biotechnology*, 11(2):117-128 (1999); and Bönig, *et al.*, *Bone Marrow Transplantation*, 28:259-264 (2001). An exemplary conjugate of G-CSF is prepared as discussed above for the conjugate of the interferons. One of skill in the art will appreciate that many other  
20 proteins may be conjugated to interferon using the methods and compositions of the invention, including but not limited to, the peptides listed in Tables 7 and 8 (presented elsewhere herein) and Figure 28, and in Figures 29-57, where individual modification schemes are presented.

In still a further exemplary embodiment, there is provided a conjugate with biotin.  
25 Thus, for example, a selectively biotinylated peptide is elaborated by the attachment of an avidin or streptavidin moiety bearing one or more modifying groups.

In a further preferred embodiment, the biomolecule is selected to direct the peptide modified by the methods of the invention to a specific intracellular compartment, thereby enhancing the delivery of the peptide to that intracellular compartment relative to the amount  
30 of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized peptide delivered to a specific intracellular

compartment within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about 100%. In another particularly preferred embodiment, the biomolecule is linked to the peptide by a cleavable linker that can hydrolyze once internalized. Presently, preferred biomolecules for intracellular targeting applications include transferrin, lactotransferrin (lactoferrin), melanotransferrin (p97), ceruloplasmin, and divalent cation transporter, as well as antibodies directed against specific vascular targets. Contemplated linkages include, but are not limited to, protein-sugar-linker-sugar-protein, protein-sugar-linker-protein and multivalent forms thereof, and protein-sugar-linker-drug where the drug includes small molecules, peptides, lipids, among others.

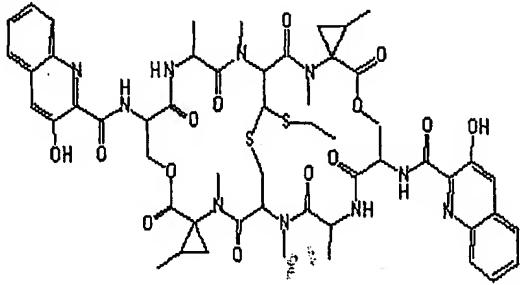
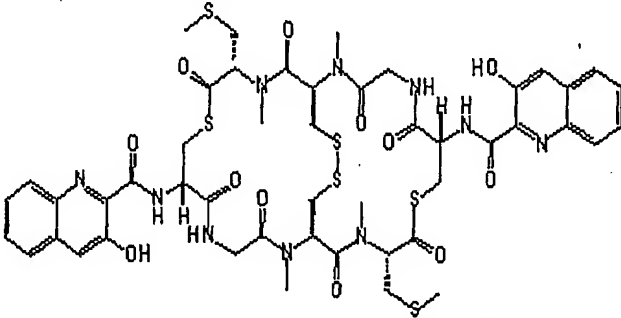
Site-specific and target-oriented delivery of therapeutic agents is desirable for the purpose of treating a wide variety of human diseases, such as different types of malignancies and certain neurological disorders. Such procedures are accompanied by fewer side effects and a higher efficiency of drug. Various principles have been relied on in designing these delivery systems. For a review, see Garnett, *Advanced Drug Delivery Reviews* 53:171-216 (2001).

One important consideration in designing a drug delivery system to target tissues specifically. The discovery of tumor surface antigens has made it possible to develop therapeutic approaches where tumor cells displaying definable surface antigens are specifically targeted and killed. There are three main classes of therapeutic monoclonal antibodies (antibody) that have demonstrated effectiveness in human clinical trials in treating malignancies: (1) unconjugated MAb, which either directly induces growth inhibition and/or apoptosis, or indirectly activates host defense mechanisms to mediate antitumor cytotoxicity; (2) drug-conjugated MAb, which preferentially delivers a potent cytotoxic toxin to the tumor cells and therefore minimizes the systemic cytotoxicity commonly associated with conventional chemotherapy; and (3) radioisotope-conjugated MAb, which delivers a sterilizing dose of radiation to the tumor. See review by Reff et al., *Cancer Control* 9:152-166 (2002).

In order to arm MAbs with the power to kill malignant cells, the MAbs can be connected to a toxin, which may be obtained from a plant, bacterial, or fungal source, to form chimeric proteins called immunotoxins. Frequently used plant toxins are divided into two

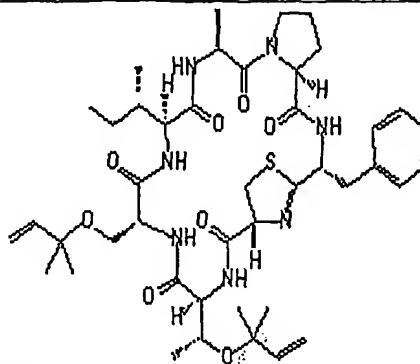
classes: (1) holotoxins (or class II ribosome inactivating proteins), such as ricin, abrin, mistletoe lectin, and modeccin, and (2) hemitoxins (class I ribosome inactivating proteins), such as pokeweed antiviral protein (PAP), saporin, Bryodin 1, bouganin, and gelonin. Commonly used bacterial toxins include diphtheria toxin (DT) and *Pseudomonas* exotoxin (PE). Kreitman, *Current Pharmaceutical Biotechnology* 2:313-325 (2001). Other toxins contemplated for use with the present invention include, but are not limited to, those in Table 2.

Table 2. Toxins.

Chemical Structure				
Toxin Name/ Source/ Alternate ID	CAS RN / Analog	Indication/ Toxicity	Mechanism	Activity (IC <sub>50</sub> nM); Tumor Type
				
SW-163E/ <i>Streptomyces</i> sp SNA 15896/ SW-163E	260794-24-9; 260794-25-0/ SW-163C; SW-163A; SW-163B	Cancer and Antibacterial/ low toxicity (mice ip)	not reported	0.3 P388 0.2 A2780 0.4 KB 1.6 colon 1.3 HL-60
				
Thiocoraline/ <i>Micromonospora marina</i> (actinomycete)	173046-02-1	Breast Cancer; Melanoma; Non-small lung cancer / not reported	DNA Polymerase alpha inhibitor (blocks cell progression from G1 to	lung, colon, CNS melanoma



S)

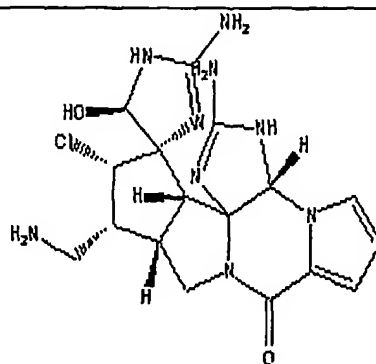


**Trunkamide A<sup>1</sup>**  
*Lissoclinum* sp (aascidian)

181758-83-8

Cancer/  
not reported

not reported cell culture (IC<sub>50</sub> in  
micrograms/mL);  
0.5 P388;  
0.5 A549;  
0.5 HT-29;  
1.0 MEL-28

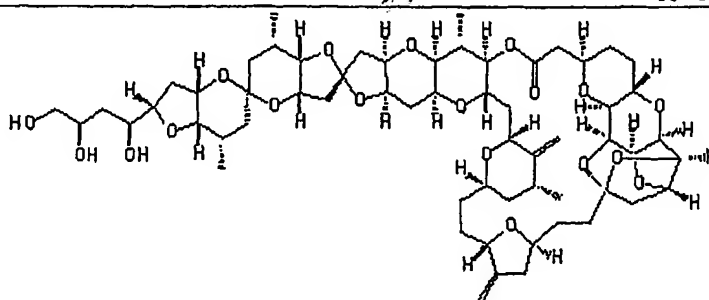


**Palauamine<sup>2</sup>**  
*Stylotella agminata*  
(sponge)

148717-58-2

Lung cancer/  
LD<sub>50</sub> (i.p. in mice) is 13  
mg/Kg

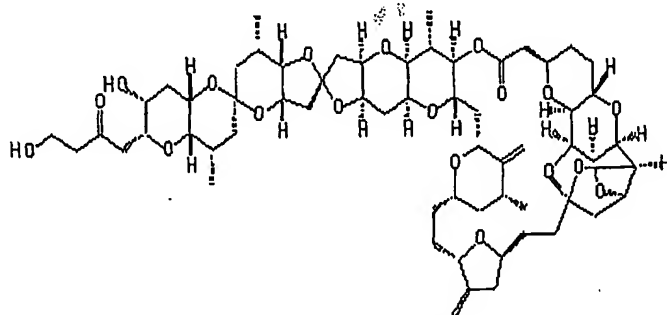
not reported cell culture (IC<sub>50</sub> in  
micrograms/mL);  
0.1 P388  
0.2 A549 (lung)  
2 HT-29 (colon)  
10 KB



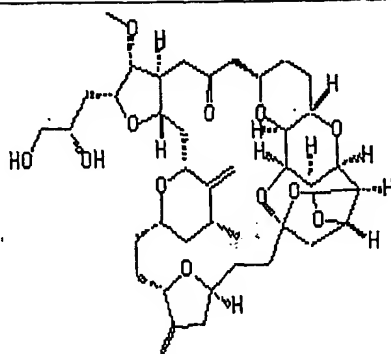
**Halichondrin B**/  
*Halichondria Okadai*,  
*Axinella Carteri* and  
*Phanell Carteri*  
(sponges)/  
NSC-609385

103614-76-2/ cancer/  
isohomohalic myelotoxicity dose  
hondrin B limiting (dogs, rats)

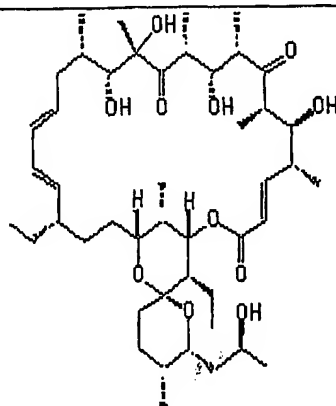
antitubulin; NCI tumor panel;  
cell cycle GI<sub>50</sub> from 50 nM to  
inhibitor 0.1 nM;  
(inhibits LC<sub>50</sub>'s from 40 μM to  
GTP binding 0.1 nM (many 0.1 to 25  
to tubulin) nM)



<b>Isohomo-halichondrin B/</b> <i>Halichondria Okadai,</i> <i>Axinell Carteri</i> and <i>Phanikell carteri</i> (sponges)/ NSC-650467	157078-48-3/ halichondrin B	melanoma, lung, CNS, colon, ovary/ not reported	antitubulin; cell cycle inhibitor (inhibits GTP binding to tubulin)	IC50's in 0.1 nM range (NCI tumor panel)
---	-----------------------------------	---	--	---



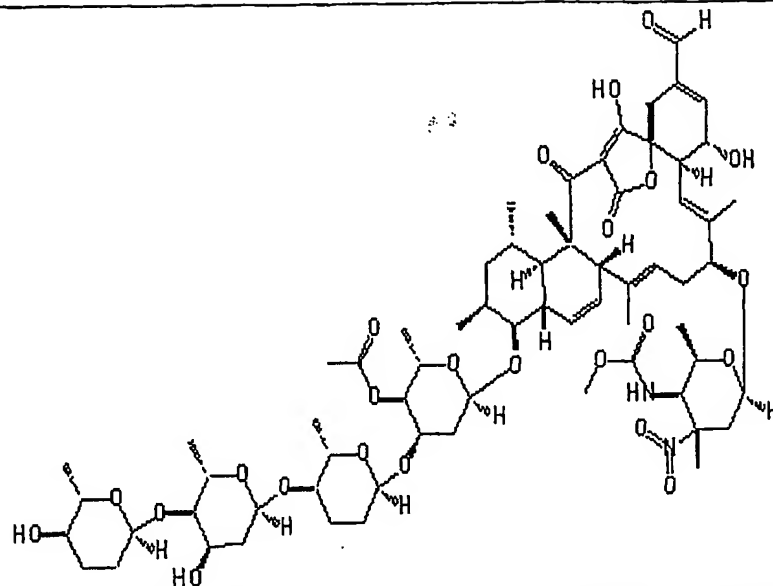
<b>Halichondrin B analogs/</b> semi-synthetic starting from <i>Halichondria</i> <i>Okadai</i> , <i>Axinell Carteri</i> and <i>Phanikell carteri</i> (sponges)/ ER-076349; ER-086526; B-1793; E-7389	253128-15-3/ ER-076349; ER-086526; B-1793; E-7389	solid tumors/ not reported	tubulin binding agent; disruption of mitotic spindles	cell culture (not reported); animal models active (tumor regression observed) in lymphoma, colon (multi-drug resistant).
--	---	-------------------------------	--	--



<b>NK-130119/</b> <i>Streptomyces</i>	132707-68-7	antifungal and anticancer/	not reported	25 ng/mL colon 8.5 ng/mL lung
--	-------------	-------------------------------	--------------	----------------------------------

*bottropensis*/  
NK-130119

not reported



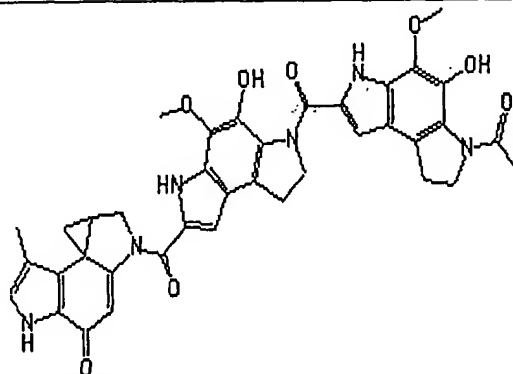
**Tetrocarcin A**/  
not reported/  
KF-67544

73666-84-9/  
analogs are  
reported

cancer/  
not reported

inhibits the  
anti-  
apoptotic  
function of  
Bcl2

not reported



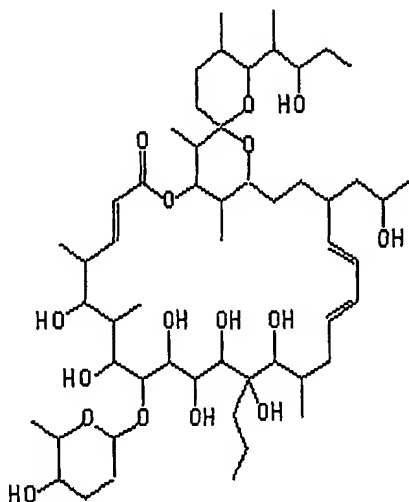
**Gilvusmycin**/  
*Streptomyces* QM16

195052-09-6

cancer/  
not reported

not reported

IC50's in ng/mL:  
0.08 P388  
0.86 K562 (CML)  
0.72 A431 (EC)  
0.75 MKN28 (GI);  
(for all < 1 nM)

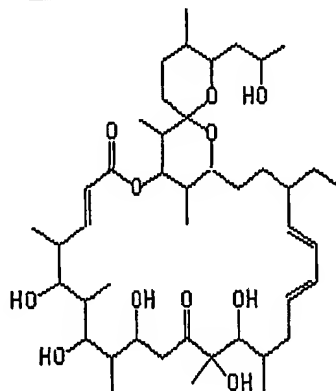


IB-96212/  
marine actinomycete/  
IB-96212

220858-11-7/  
IB-96212;  
IB-98214;  
IB-97227

Cancer and  
Antibacterial/  
not reported

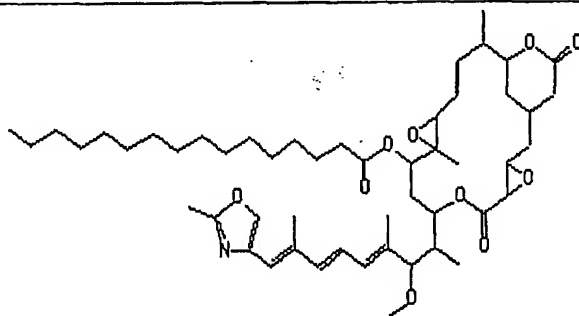
not reported IC50's in ng/mL:  
0.1 P388



BE-56384<sup>3</sup>/  
*Streptomyces* Sp./  
BE-56384

207570-04-5 cancer/  
not reported

not reported IC50's in ng/mL:  
0.1 P388  
0.29 colon 26  
34 DLD-1  
0.12 PC-13  
0.12 MKM-45



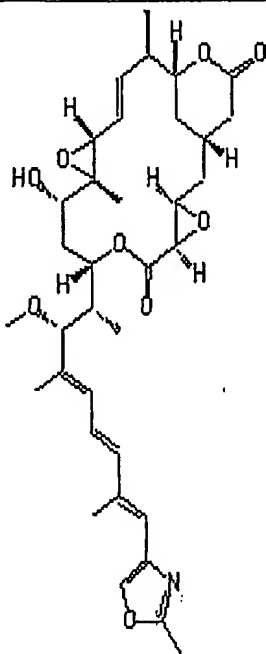
Palmitoylrhizoxin/

135819-69-1/ cancer/

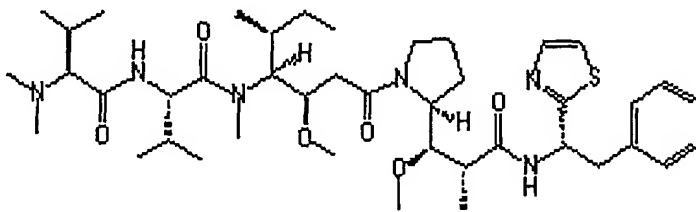
tubulin

not reported

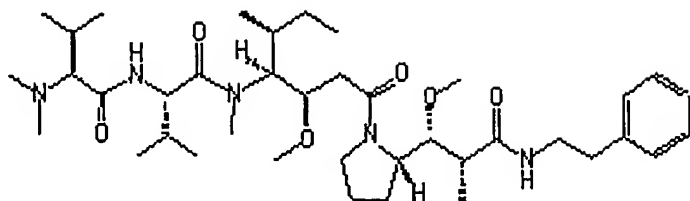
semi-synthetic; <i>Rhizopus chinensis</i>	Analog of rhizoxin	binds LDL; less cytotoxic than rhizoxin	binding agent (cell cycle inhibitor)
---	--------------------	---	--------------------------------------



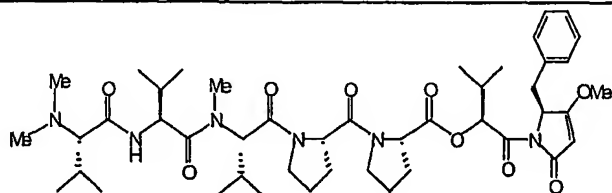
<b>Rhizoxin/</b> <i>Rhizopus chinensis</i> / WF-1360; NSC-332598; FR-900216	95917-95-6; 90996-54-6	melanoma, lung, CNS, colon, ovary, renal, breast, head and neck/ Rapid Drug clearance; High AUC correlates with high toxicity	tubulin binding agent (cell cycle inhibitor)	NCI tumor panel (NSC 332598); log GI50's: 50 nM to 50 fM; log LC50's: 50 μM to 0.5 nM (several cell lines at 50 fM).
--	---------------------------	--	--	---



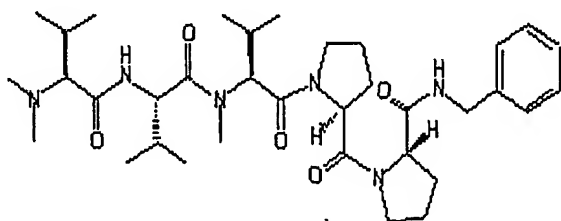
<b>Dolastatin-10/</b> <i>Dolabella auricularia</i> (sea hare)/ NSC-376128	110417-88-4/ other Dolistatins (ie. 15) and analog	prostate, melanoma, leukemia/ myelotoxicity (at greater than 0.3 pM)	tubulin binding (tubulin aggregation)	NCI tumor panel (60 cell line; GI50); 25 nM to 1 pM (most < 1 nM) (three cell lines μM)
--	--	---	--	---



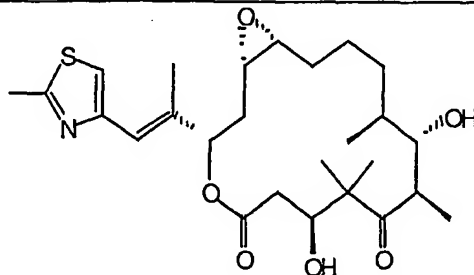
soblidotin/ synthetic/ TZT-1027; auristatin PE	149606-27-9/ analog prepared	cancer (pancreas, esophageal colon, breast, lung, etc) / MTD was 1.8 mg/Kg (IV); toxicity not reported	tubulin binding agent	cell culture: colon, melanoma, M5076 tumors, P388 with 75- 85% inhibition (dose not reported)
--	------------------------------------	---	-----------------------------	---



Dolastatin-15/ <i>Dolabella auricularia</i> (sea hare)	not reported/ other Dolastatins (ie. 15) and analog	cancer/ not reported	Tubulin binding (tubuline aggregation)	NCI tumor panel (60 cell line; GI50); 25 nM to 39 pM (most < 1 nM) (one cell line 2.5 μM); most active in breast
--	---	-------------------------	---	---

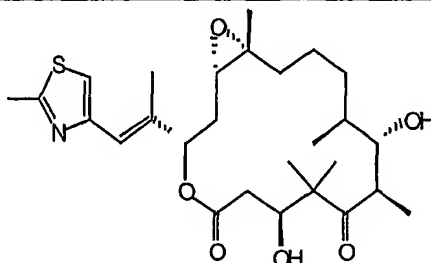


Cemadotin <sup>4</sup> / Synthetic; Parent Dolastatin-15 was isolated from <i>Dolabella</i> <i>auricularia</i> (sea hare)/ LU-103793; NSC D- 669356	1159776-69- 9/ many analogs	melanoma/ hypertension, myocardial ischemia and myelosuppression were dose-limiting toxicities.	tubulin binding (tubulin aggregation)	NCI tumor panel (NCS D-669356); active in breast, ovary, endometrial, sarcomas and drug resistant cell lines. Data not public.
---	-----------------------------------	---	--	---

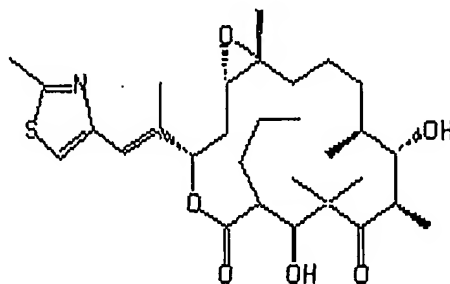


Epothilone A/	not reported/	cancer/	tubulin	IC50's of;
---------------	---------------	---------	---------	------------

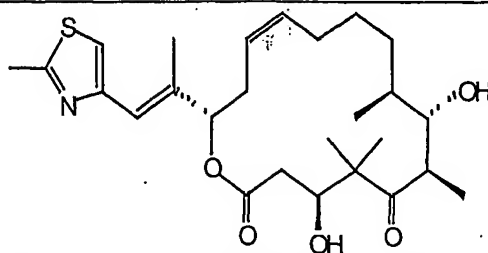
Synthetic or isolated from many analogs not reported	binding	1.5 nM MCF-7 (breast)
<i>Sorangium cellulosum</i>	(tubulin	27.1 nM MCF-7/ADR
(myxococcales) strain	polymeriza-	2.1 nM KB-31
So ce90)	tion)	(melanoma)
		3.2 nM HCT-116



<b>Epothilone B/</b>	152044054-7/	Solid tumors (breast,	tubulin	IC50's of;
Synthetic or isolated from many analogs		ovarian, etc)/	binding	0.18 nM MCF-7
<i>Sorangium cellulosum</i>		well tolerated; t1/2 of	(tubulin	(breast)
(myxococcales) strain So		2.5 hrs; partial	polymeriza-	2.92 nM MCF-7/ADR
ce90) /		responses (phase I);	tion)	0.19 nM KB-31
EPO-906		diarrhea major side		(melanoma)
		effect.		0.42 nM HCT-116;
				broad activity reported



<b>Epothilone Analog /</b>	not reported /	cancer/	tubulin	IC50's of 0.30 to
Synthetic or semi-	hundreds of	not reported	binding	1.80 nM in various
synthetic; Original lead,	analog		(tubulin	tumor cell lines;
Epothilone A, isolated			polymeriza-	active in drug resistant
from <i>Sorangium</i>			tion)	cell lines
<i>cellulosum</i>				
(myxococcales) strain So				
ce90)/				
ZK-EPO				



<b>Epothilone D /</b>	189452-10-9/	Solid tumors (breast,	tubulin	NCI tumor panel (NSC-
Epothilone D, isolated	many analogs	ovarian, etc)/	binding	703147; IC50);
from <i>Sorangium</i>		emesis and anemia; t1/2	(tubulin	0.19 nM KB-31
<i>cellulosum</i>		of 5-10 hrs.	polymeriza-	(melanoma)

(myxococcales) strain So  
ce90)/  
KOS-862

tion)

0.42 nM HCT-116;  
broad activity reported

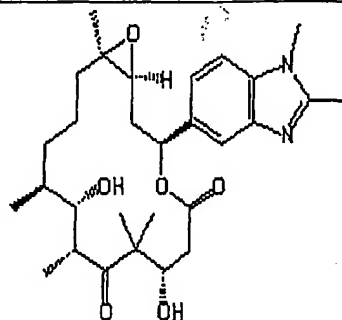
### Structure Not Identified

**Epothilone D analog <sup>5/</sup>**  
Synthetic or semi-  
synthetic; Original lead,  
Epothilone D, isolated  
from *Sorangium*  
*cellulosum*  
(myxococcales) strain So  
ce90)/  
KOS-166-24

189453-10-9/ Solid tumors;  
hundreds of not reported  
analogs

tubulin  
binding  
(tubulin  
polymeriza-  
tion)

not reported

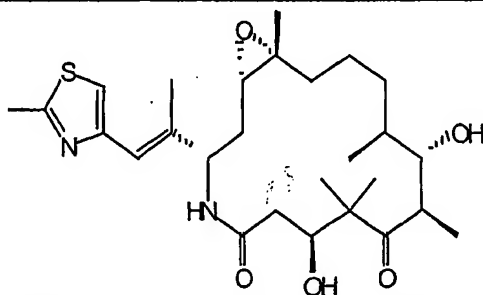


**Epothilone Analog /**  
Synthetic; Original lead,  
Epothilone A, isolated  
from *Sorangium*  
*cellulosum*  
(myxococcales) strain So  
ce90)/  
CGP-85715

not reported/ cancer;  
hundreds of not reported  
analogs

tubulin  
binding  
(tubulin  
polymeriza-  
tion)

not reported



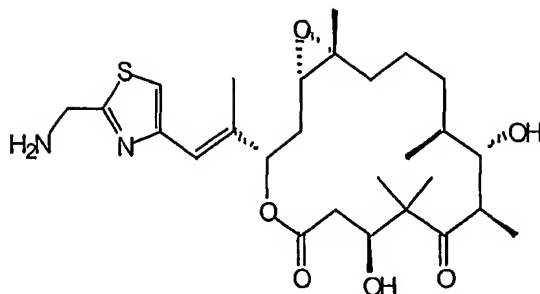
**Epothilone Analog/**  
Synthetic or semi-  
synthetic; Original lead,  
Epothilone B, isolated  
from *Sorangium*  
*cellulosum*  
(myxococcales) strain So  
ce90)/  
BMS-247550

219989-84-1/ non-small cell Lung,  
hundreds of breast, stomach tumor  
analogs (objective responses in  
breast ovarian and lung)/  
sever toxicity (fatigue,  
anorexia, nauseas,  
vomiting, neuropathy  
myalgia)

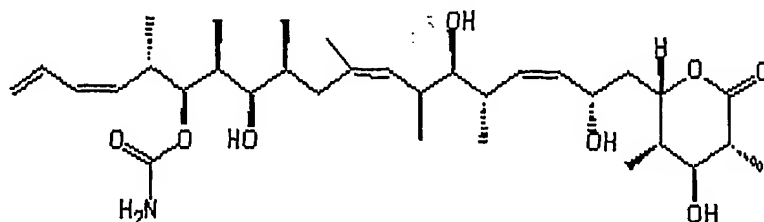
tubulin  
binding  
(tubulin  
polymerizati  
on)

NCI tumor Panel (NSC-  
710428 & NSC-  
710468); 8-32 nM  
(NCI data not available)

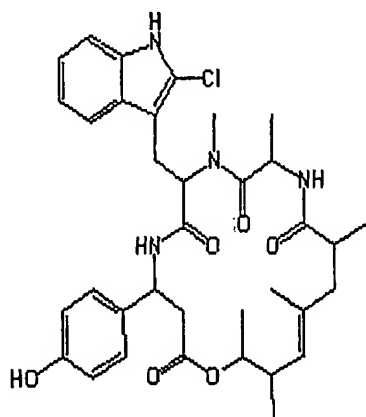




<b>Epothilone Analog /</b> Synthetic or semi-synthetic; Original lead, Epothilone B, isolated from <i>Sorangium cellulosum</i> (myxococcales) strain So ce90/ BMS-310705	not reported/ hundreds of analogues	advanced cancers/ adverse events (diarrhea, nausea, vomiting, fatigue, neutropenia); t1/2 of 3.5 hrs; improved water solubility to BMS 247550.	tubulin binding (tubulin polymerization)	broad activity with IC50's of 0.7 to 10 nM
--	---	---	--	---



<b>Discodermolide /</b> synthetic; originally isolated from <i>Discodermia dissoluta</i> (deep water sponge); rare compound (7 mg per 0.5 Kg sponge/ XAA-296	127943-53-7/ analogues less potent	solid tumors/ not reported; 100-fold increase in water solubility over taxol	tubulin stabilizing agent (similar to taxol)	Broad activity (A549-nonslung, prostate, P388, ovarian with IC50's about 10 nM) including multi-drug resistant cell lines;
--	--	---	---	--

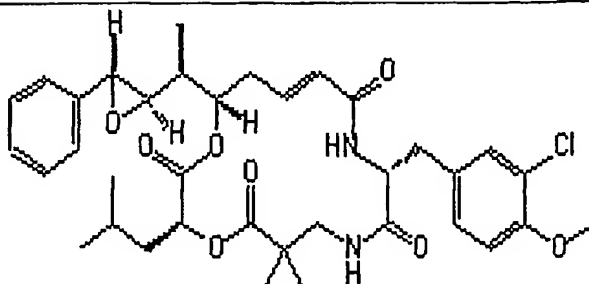


<b>Chondramide D/</b>	172430-63-6	cancer/	tubulin	5 nM A-549
-----------------------	-------------	---------	---------	------------

not reported

not reported

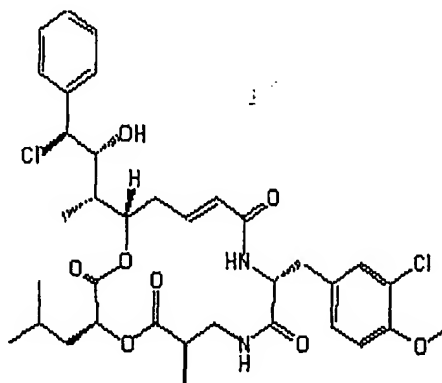
binding (epidermoid carcinoma)  
 agent; actin 15 nM A-498 (kidney)  
 polymeriza- 14 nM A549 (lung)  
 tion inhibitor 5 nM SK-OV-3 (ovary)  
 3 nM U-937  
 (lymphoma)



**Cryptophycin analogs**  
 (including 52, 55 and  
 others)<sup>6/</sup>  
*Nostoc* sp GSV 224 (blue-  
 green algae) isolated  
 Cryptophycin 1/  
 LY-355703; Ly-355702;  
 NSC-667642

204990-60-3 solid tumors, colon  
 and 186256- cancer/  
 67-7/ Phase II studies halted  
 many potent because of severe  
 analogs toxicity with one death  
 prepared at resulting from drug;  
 Lilly

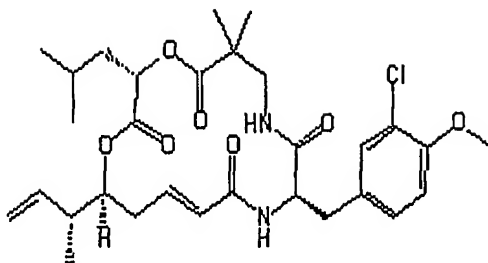
tubulin broad activity (lung,  
 polymeriza- breast, colon, leukemia)  
 tion inhibitor with IC50's of 2 to 40  
 pM; active against  
 multi-drug resistance  
 cell lines (resistant to  
 MDR pump). NCI  
 tumor panel, GI50's  
 from 100 nM to 10 pM;  
 LC50's from 100 nM to  
 25 pM.



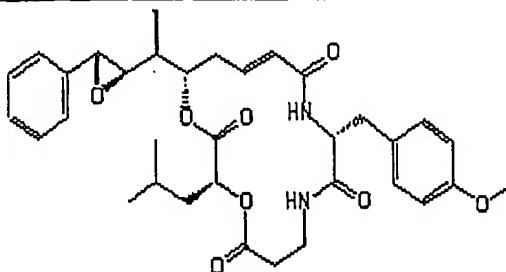
**Cryptophycin 8/**  
 semi-synthetic; starting  
 material from *Nostoc* sp.

168482-36-8; solid tumors/  
 168482-40-4; not reported  
 18665-94-1;  
 124689-65-2;  
 125546-14-7/  
 cryptophycin  
 5, 15 and 35

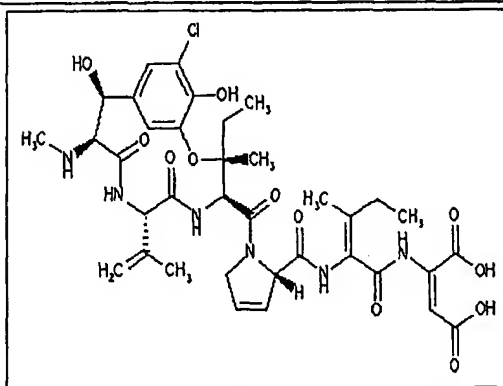
tubulin broad spectrum  
 polymeriza- anticancer activity (cell  
 tion inhibitor culture) including  
 multi-drug resistant  
 tumors



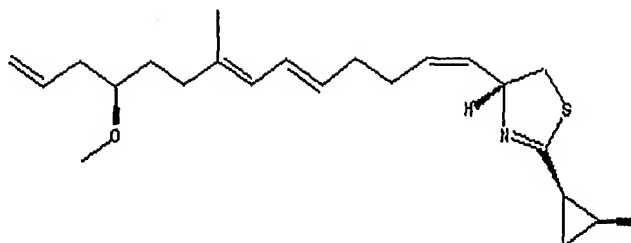
**Cryptophycin analogs<sup>7</sup>** 219660-54-5/ solid tumors/ topoisomer- not reported  
 synthetic; semi-synthetic, LY-404292 not reported ase inhibitors  
 starting material from  
*Nostoc* sp./  
 LY-404291



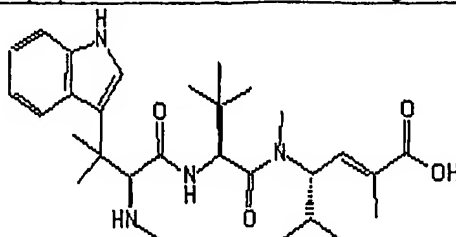
**Arenastatin A analogs<sup>8</sup>** not reported/ cancer/ inhibits 8.7 nM (5 pg/mL) KB  
*Dysidea arenaria* (marine analogs not reported tubulin (nasopharyngeal); NCI  
 sponge)/ prepared polymeriza- tumor panel (GI50's);  
 Cryptophycin B; NSC- tion 100 pM to 3 pM  
 670038



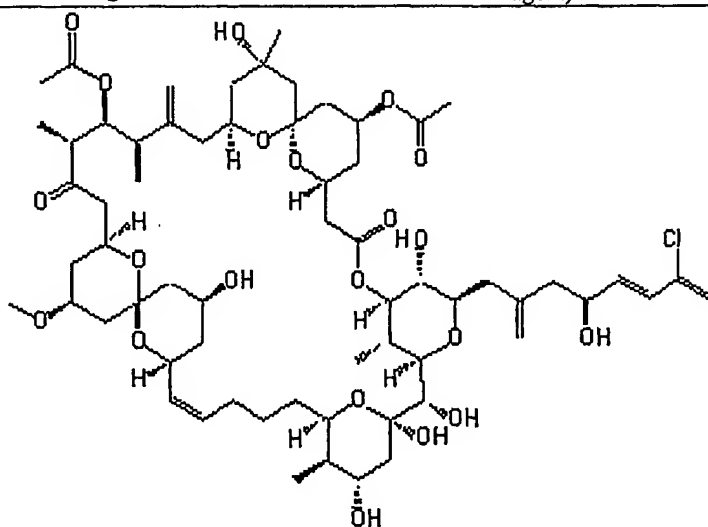
**Phomopsin A/** not reported Liver cancer (not as tubulin potent anticancer  
*Diaporthe toxicus* or potent in other cancers)/ binding activity especially  
*Phomopsis* not reported agent against liver cancer  
*leptostromiformis* (fungi)



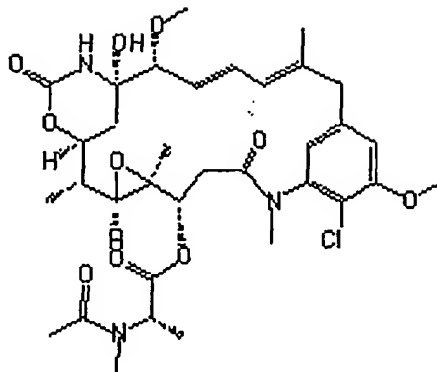
<b>Curacin A and analogs/</b> <i>Lyngbya majuscula</i> (blue green cyanobacterium)	155233-30-0/ analogs have been prepared	Cancer/ not reported	Tubulin binding agent	broad activity (cancer cell lines); 1-29 nM
--	---	-------------------------	-----------------------------	--



<b>Hemiasterlins A &amp; B and analogs<sup>9</sup>/</b> <i>Cymbastela</i> sp.	not reported/ criamide A & B; geodiamiolid- G	Cancer/ not reported	Antimitotic agent (tubulin binding agent)	broad activity: 0.3-3 nM MCF7 (breast); 0.4 ng/mL P388
--	---	-------------------------	---	---



<b>Spongistatins (1-9)<sup>10</sup>/</b> <i>Spirastrell spinispirulifera</i> (sea sponge)	149715-96-8; 158734-18-0; 158681-42-6; 158080-65-0; 150642-07-2; 153698-80-7; 153745-94-9; 150624-44-5; 158734-19-1/ other spongistatins	cancer/ not reported	tubulin binding agent	Most potent compounds ever tested in NCI panel cell line (mean GI50's of 0.1 nM; Spongistatin-1 GI50's of 0.025-0.035 nM with extremely potent activity against a subset of highly chemoresistant tumor types
---	--	-------------------------	-----------------------------	---



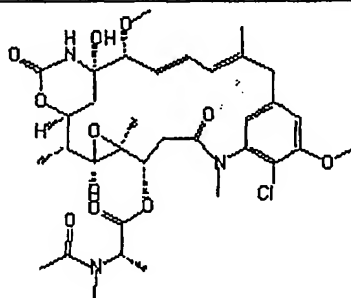
Maytansine/  
*Maytenus* sp./  
NSC-153858

35846-53-8/  
other related  
macrolides

cancer/  
severe toxicity

tubulin  
binding  
agent (causes  
extensive  
disassembly  
of the  
microtubule  
and totally  
prevents  
tubulin  
spiralization)

Broad Activity in NCI  
tumor panel (NSC-  
153858; NSC-153858);  
NCI tumor panel,  
GI50's from 3  $\mu$ M to  
0.1 pM; LC50's from  
250  $\mu$ M to 10 pM. Two  
different experiments  
gave very different  
potencies.



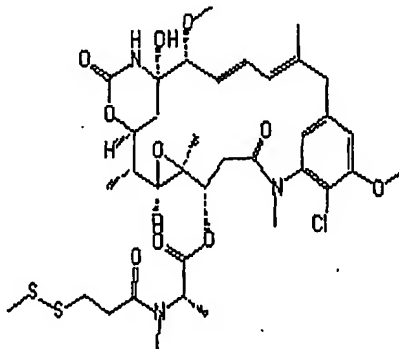
Maytansine-IgG(EGFR  
directed)-conjugate<sup>11</sup>/  
semi-synthetic; starting  
material from *Maytenus*  
sp.

not reported/  
other related  
macrolides

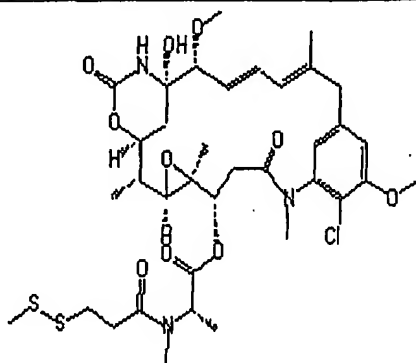
breast, head and neck,  
Squamous cell  
carcinoma/  
not reported

EGFR  
binding and  
tubulin  
binding

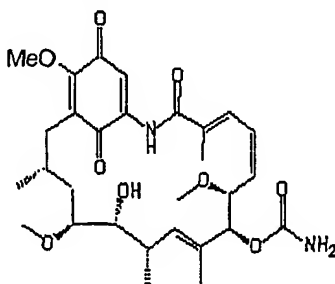
not reported



Maytansine-IgG(CD56 antigen)-conjugate <sup>12</sup> , 3.5 drug molecules per IgG/ semi-synthetic; starting material from <i>Maytenus</i> sp./ huN901-DM1	not reported/ other related macrolides	Neuroendocrine, small-cell lung, carcinoma/ mild toxicity (fatigue, nausea, headaches and mild peripheral neuropathy); no hematological toxicity; MTD 60 mg/Kg, I.V., weekly for 4 weeks; only stable disease reported (humans)	CD56 binding and tubulin binding	antigen-specific cytotoxicity (cell culture; epidermal, breast, renal ovarian colon) with IC <sub>50</sub> 's of 10-40 pM; animal studies (mice SCLC tumor--alone and in combination with taxol or cisplatin completely eliminated tumors).
---	--	---	----------------------------------	---

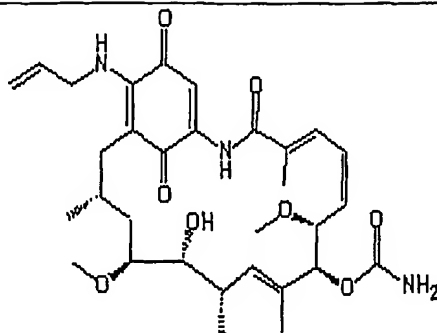


Maytansine-IgG(CEA antigen)-conjugate <sup>13</sup> , 4 drug molecules per IgG/ semi-synthetic; starting material from <i>Maytenus</i> sp./ C424-DM1	not reported/ other related macrolides	non-small-cell lung, carcinoma pancreas, lung, colon/ mild toxicity (fatigue, nausea, headaches and mild peripheral neuropathy); pancreatic lipase elevated; MTD 88 mg/Kg, I.V., every 21 days; only stable disease reported (humans); t <sub>1/2</sub> was 44 hr.	CEA binding and tubulin binding	antigen-specific cytotoxicity (cell culture; epidermal, breast, renal ovarian colon) with IC <sub>50</sub> 's of 10-40 pM; animal studies (mice: melanoma [COLO-205]--alone and in combination with taxol or cisplatin completely eliminated tumors);
--	--	--	---------------------------------	---

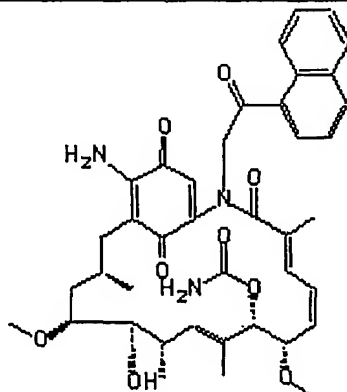


Geldanamycin / <i>Streptomyces hygroscopicus</i> var. Geldanus/ NSC-212518; Antibiotic U 29135; NSC-122750	30562-34-6/ natural derivatives	cancer/ not reported	binds Hsp 90 chaperone and inhibits function	NCI tumor panel (cell culture); 5.3 to 100 nM; most active in colon, lung and leukemia. NCI tumor panel, GI <sub>50</sub> 's from 10 μM to 0.1 nM; LC <sub>50</sub> 's from 100 μM to 100
--	---------------------------------	----------------------	--	---

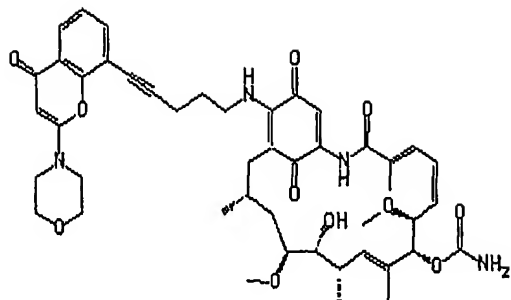
nM. Two assays with  
very different potencies.



<b>Geldanamycin Analog/</b> semi-synthetic; / CP-127374; 17-AAG; NSC-330507	745747-14-7/ Kosan, NCI and UK looking for analogs with longer t1/2 and oral activity; analogs include: NSC- 255110; 682300; 683661; 683663.	solid tumors/ Dose limiting toxicities (anemia, anorexia, diarrhea, nausea and vomiting); t1/2 (i.v.) is about 90 min; no objective responses measured at 88 mg/Kg (i.v. daily for 5 days, every 21 days);	binds Hsp 90 chaperone and inhibits function	cell culture (not reported); animal models active (tumor regression observed) in breast, ovary, melanoma, colon.
--	---	---	---	---



<b>Geldanamycin analog/</b> semi-synthetic; / CP-202567	not reported/ analogs prepared	solid tumors/ not reported	binds Hsp 90 chaperone and inhibits function	not reported
---	--------------------------------------	-------------------------------	---	--------------



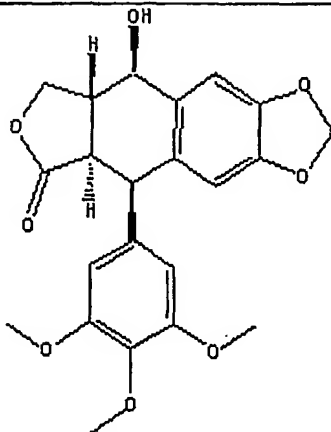
<b>Geldanamycin conjugates/</b> semi-synthetic; / LY-294002-GM; PI3K-1-GM	345232-44-2/ analogs prepared	breast/ not reported	binds Hsp 90 chaperone and inhibits function; binds and inhibits PI-3 kinase	cell culture (no reported); animal models performed
---	-------------------------------------	-------------------------	--	---

## Structure Not Reported

<b>Geldanamycin Analog/</b> not reported/ CNF-101	not reported/ analogs prepared	breast, prostate/ not reported	binds Hsp 90 chaperone and inhibits function	not reported
---	--------------------------------------	-----------------------------------	---	--------------

## Structure Not Reported

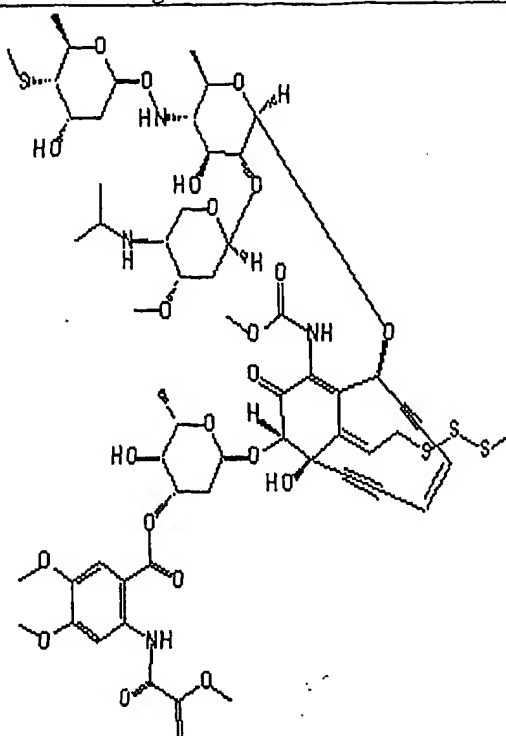
<b>Geldanamycin-testosterone conjugate/</b> semi-synthetic/ GMT-1	not reported/ analogs prepared	prostate/ not reported	binds Hsp 90 chaperone and inhibits function and testosterone receptors where it is internalized	not reported; conjugate has a 15-fold selective cytotoxicity for androgen positive prostate cells
---	--------------------------------------	---------------------------	---	---



<b>Podophyllotoxin/</b> <i>Podophyllum</i> sp.	518-28-5/ many analogs	Verruca vulgaris, Condyloma/ severe toxicity when	tubulin inhibitor and topoisomer-	broad activity (cell culture) with IC50's in μM range
---	---------------------------	---	---	---



given i.v. or s.c.      ase inhibitor



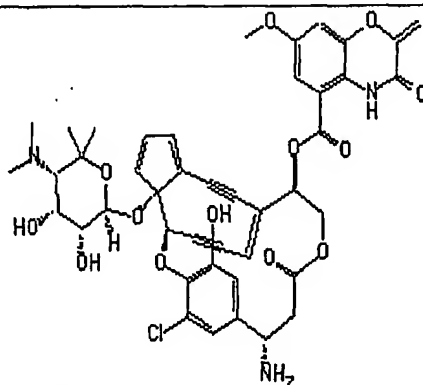
esperamicin-A1/  
not known/  
BBM-1675A1; BMY-  
28175; GGM-1675

99674-26-7

cancer/  
not reported (suspected  
severe toxicity)

DNA  
cleaving  
agent

highly potent activity  
(cell culture); animal  
models highly potent  
with optimal dose of  
0.16 micrograms/Kg



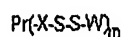
C-1027<sup>14</sup>/  
*Streptomyces setonii* C-  
1027/  
C-1027

120177-69-7

cancer (examined  
hepatoma, breast, lung  
and leukemia/  
not reported

DNA  
cleaving  
agent

extremely potent (cell  
culture) IC<sub>50</sub>'s in pM  
and fM; conjugated to  
antibodies the potency  
remains the same (ie.  
5.5 to 42 pM);



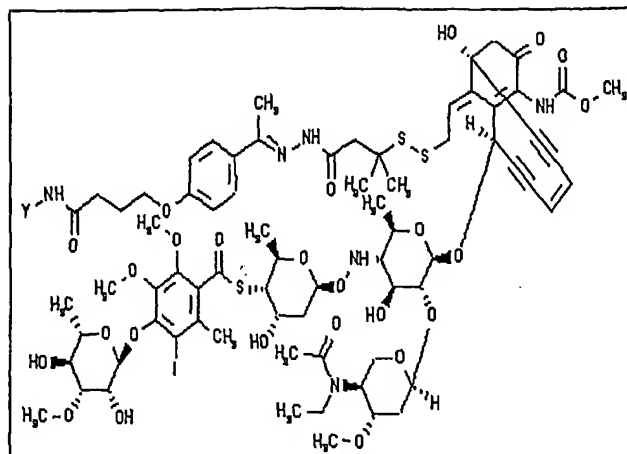
$m = 0.5 - 15$

Pr = proteinaceous carrier

W = calicheamicin minus Me-S-S-S

X = linker

Y = antibody P76.6

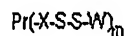


**Calicheamicin-IgG(CD33 antigen)-conjugate<sup>15</sup>**  
 semi-synthetic;  
*Micromonospora echinospora*  
 gemtuzumab ozogamicin;  
 mylotarg; WAY-CMA-676; CMA-676; CDP-771

113440-58-7; AML/  
 220578-59-6/ mild toxicity  
 several  
 reported in  
 patents

DNA  
 cleaving  
 agent

Kills CD33+ cells (HL-60, NOMO-1, and NKM-1) at 100 ng/mL; MDR cell lines are not effected by the drug.



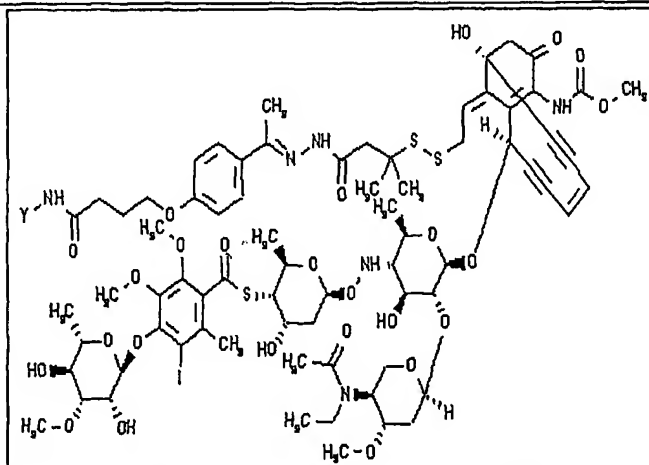
$m = 0.5 - 15$

Pr = proteinaceous carrier

W = calicheamicin minus Me-S-S-S

X = linker

Y = antibody P76.6

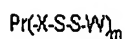


**Calicheamicin-IgG-conjugates<sup>16</sup>**  
 semi-synthetic;  
*Micromonospora echinospora*

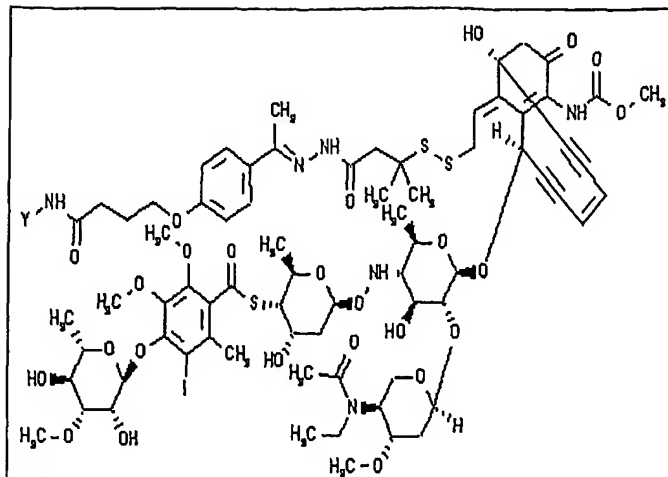
113440-58-7; cancer/  
 220578-59-6 not reported

DNA  
 cleaving  
 agent

TBD

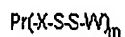


$m = 0.5 - 15$   
 Pr = proteinaceous carrier  
 W = calicheamicin minus Me-S-S-S  
 X = linker  
 Y = antibody P76.6

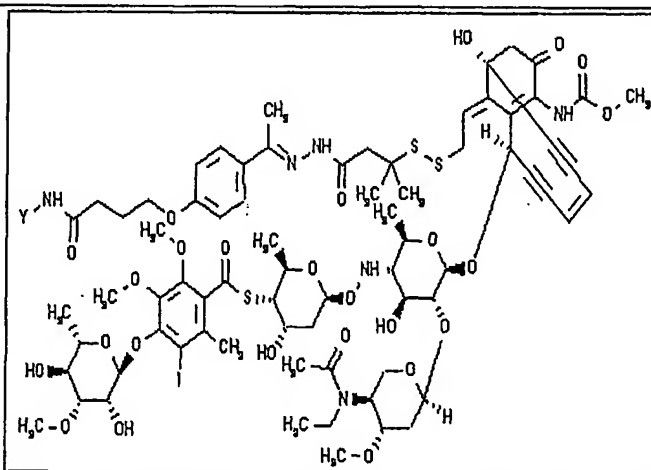


Calicheamicin-  
 IgG(OBA1 antigen)  
 conjugate/  
 semi-synthetic:  
*Micromonospora*  
*echinospora*/  
 OBA1-H8

not reported      cancer/  
                          not reported      DNA  
    cleaving  
    agent      all human cancer; data  
         not reported



$m = 0.5 - 15$   
 Pr = proteinaceous carrier  
 W = calicheamicin minus Me-S-S-S  
 X = linker  
 Y = antibody P76.6



Calicheamicin-  
 IgG(CD22 antigen)  
 conjugate/  
 semi-synthetic:  
*Micromonospora*  
*echinospora*/  
 CMC-544

not reported      non-Hodgkin lymphoma, DNA  
                          cancer/  
                          not reported      cleaving  
    agent      all human cancer; data  
         not reported

partially esterified polystyrene maleic acid copolymer (SMA)  
 conjugated to neocarzinostatin (NCS)

Neocarzinostatin<sup>17</sup>/  
 semi-synthetic;  
*Streptomyces*

123760-07-6; liver cancer and brain  
 9014-02-2 cancer/  
                          not reported

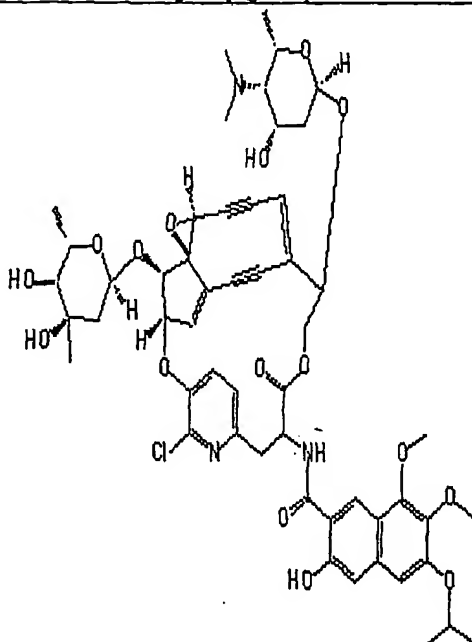
DNA  
 cleaving  
 agent

cell culture data not  
 reported.

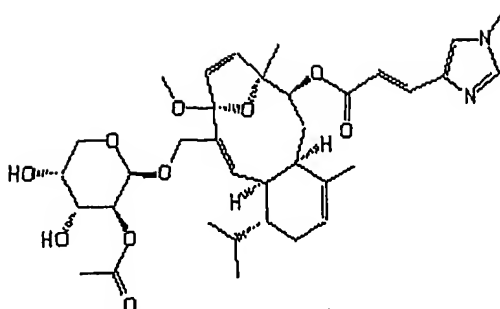
*carconistaticus*/  
Zinostatin stimalamer;  
YM-881; YM-16881

IgG (TES-23)-conjugated to neocarzinostatin

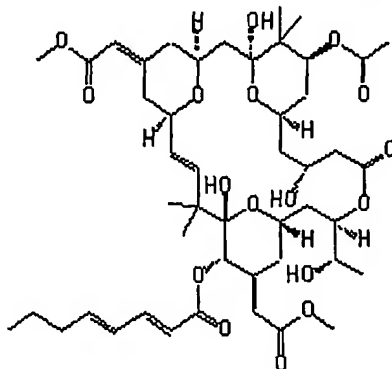
Neocarzinostatin/ not reported/ TES-23-NCS	not reported	solid tumors/ toxicity not reported; the TES-23 antibody (without anticancer agent) was as effective at eliminating tumors as the drug conjugated protein	DNA cleaving agent and immunostim- ulator	cell culture data not reported.
--	--------------	---	---	------------------------------------



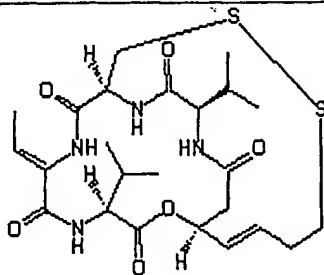
Kedarcidin <sup>18</sup> / <i>Streptoalloteichus</i> sp NOV strain L5856, ATCC 53650/ NSC-646276	128512-40-3; 128512-39-0/ chromophore and protein conjugate	cancer/ not reported	DNA cleaving agent	cell culture (IC <sub>50</sub> 's in ng/mL), 0.4 HCT116; 0.3 HCT116/VP35; 0.3 HCT116/VM46; 0.2 A2780; 1.3 A2780/DDP. animal models in P388 and B-16 melanoma. NCI tumor panel, GI <sub>50</sub> 's from 50 μM to 5 μM.
--	---	-------------------------	--------------------------	--



Eleutherobins/ marine coral	174545-76-7/ sarcodictyins (marine coral)	cancer/ not reported	tubulin binding agent	similar potency to taxol; not effective against MDR cell lines
--------------------------------	---	-------------------------	-----------------------------	--

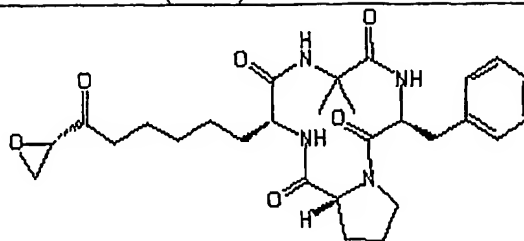


Bryostatin-1/ <i>Bugula neritina</i> (marine bryozoan)/ GMY-45618; NSC- 339555	83314-01-6	leukemia, melanoma, lung, cancer/ myalgia; accumulated toxicity; poor water solubility; dose limiting toxicity	immunostim- ulant (TNF, GMCSF, etc); enhances cell kill by current anticancer agents	not reported
--	------------	---	--	--------------

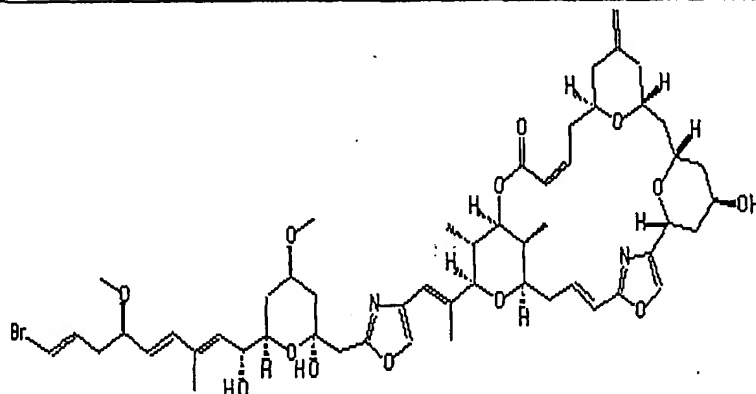


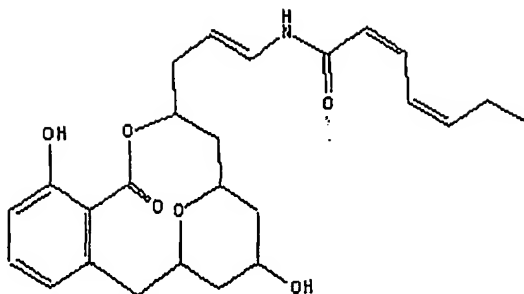
FR-901228/ <i>Chromobacterium</i> <i>violaceum</i> strain 968/ NSC-63-176; FK-228	128517-07-7	leukemia, T-cell lymphoma, cancer/ toxic doses (LD50) 6.4 and 10 mg/Kg, ip and iv respectively; GI toxicity, lymphoid atrophy; dose limiting toxicity (human) 18 mg/Kg; t1/2 of 8 hrs	histone deacetylase inhibitor	In vitro cell lines (NCI tumor panel); IC50's of between 0.56 and 4.1 nM (breast, lung, gastric colon, leukemia)
--	-------------	---	-------------------------------------	---

(human)

Chlamydocin/  
not reported

53342-16-8

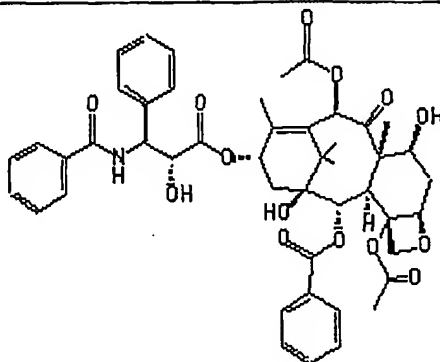
cancer/  
not reportedhistone  
deacetylase  
inhibitornot reported (cell  
culture);  
inhibits histone  
deacetylase at an IC<sub>50</sub>  
of 1.3 nMPhorboxazole A<sup>19</sup>/  
marine sponge181377-57-1; leukemia, myeloma/  
165689-31-6; not reported  
180911-82-4;  
165883-76-1/  
analogs  
preparednot reported  
(induces  
apoptosis)NCI tumor panel  
(details not reported);  
IC<sub>50</sub>'s of 1-10 nM. The  
inhibition values  
(clonogenic growth of  
human cancer cells) at  
10 nM ranged from 6.2  
to > 99.9% against  
NALM-6 human B-  
lineage acute  
lymphoblastic  
leukemia cells, BT-20  
breast cancer cells and  
U373 glioblastoma  
cells, with the specified  
compound showing  
inhibition values in the  
range of 42.4 to >  
99.9% against these cell  
lines.; IC<sub>50</sub>'s are nM  
for MDR cell lines.



Apicularen A/  
*Chondromyces robustus*

220757-06-2/  
natural  
derivatives cancer/  
not reported

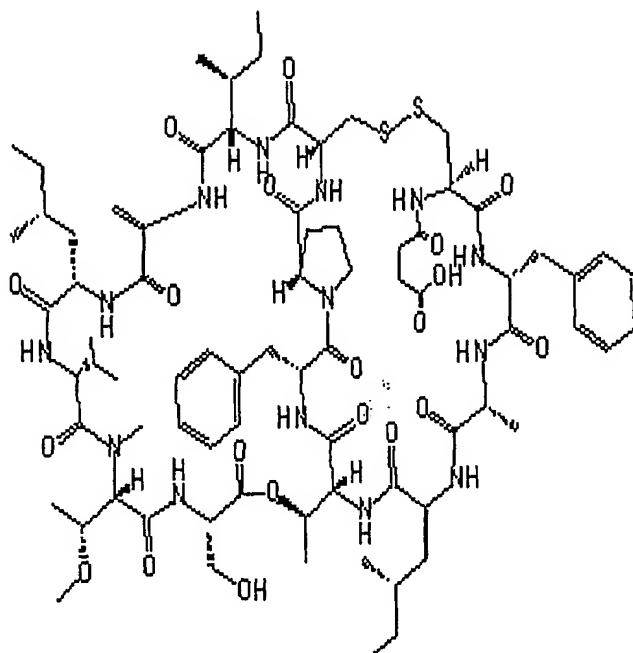
not reported IC50's of 0.1 to 3  
ng/mL (KB-3-A, KB-  
Va, K562, HL60, U937,  
A498, A549, PV3 and  
SK-OV3)



Taxol/  
Pacific yew and fungi/  
Paclitaxel; NSC-125973

33069624/  
many analogs cancer; breast, prostate,  
ovary, colon, lung, head  
& neck, etc./  
severe toxicity (grade III  
and IV) tubulin  
binding  
agent

NCI tumor panel;  
GI50's of 3 nM to 1  
 $\mu$ M;  
TGI 50 nM to 25  $\mu$ M

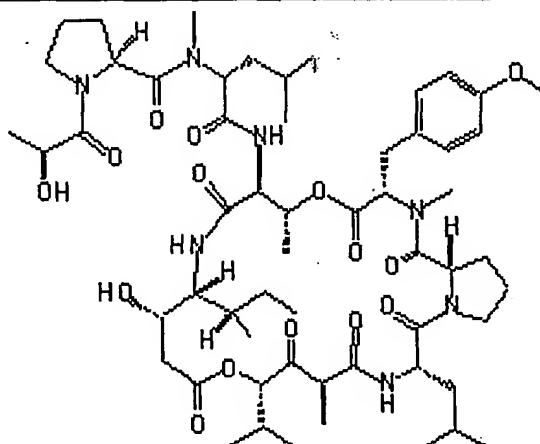


Vitilevuamide/  
*Didemnum cuculliferum*  
or *Polysyncraton*  
*lithostrotum*

191681-63-7 cancer/  
not reported

tubulin  
binding  
agent

cell culture; IC50's of  
6-311 nM (panel of  
tumor cell lines  
HCT116 cells, A549  
cells, SK-MEL-5 cells  
A498 cells). The  
increase in lifespan  
(ILS) for CDF1 mice  
after ip injection of  
P388 tumor cells was in  
the range of -45 to  
+70% over the dose  
range of 0.13 to 0.006  
mg/kg.



Didemnin B/

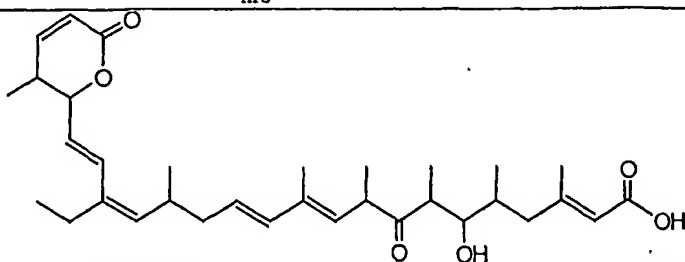
77327-05-0; non-Hodgkin's

inhibits

NCI 60-tumor panel



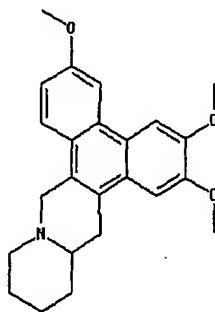
<i>Trididemnum solidum</i> / NSC-2325319; IND 24505	77327-04-9; 77327-06-1/ other related natural products	lymphoma, breast, carcinoma, CNS, colon/ Discontinued due to cardiotoxicity; nausea, neuro-muscular toxicity and vomiting MTD 6.3 mg/Kg; toxicity prevented achieving a clinically signif. effect; rapidly cleared (t1/2 4.8 hrs	protein synthesis via EF-1	(GI50's): 100 nM to 50 fM. Not potent against MDR cell lines.
---	--	--	----------------------------------	--



**Leptomycin B**/  
*Streptomyces* sp. strain  
ATS 1287/  
NSC-364372; elactocin

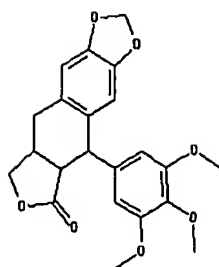
87081-35-4

NCI 60-tumor panel  
(GI50's):  
8  $\mu$ M to 1 pM; (LC50):  
250  $\mu$ M to 10 nM  
(several cell lines at 0.1  
nM). Two testing  
results with very  
different potencies.



**Cryptopleurin**/  
not known/  
NSC-19912

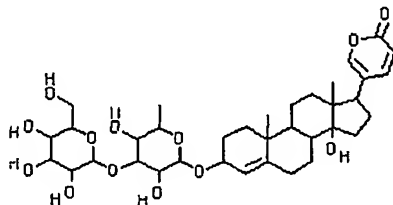
NCI 60-tumor panel  
(GI50's): 19 nM to 1  
pM; (LC50): 40  $\mu$ M to  
10 nM (several cell  
lines at 1 pM).



Sillicicolin/  
not known/  
NSC-403148,  
deoxypodophyllotoxin,  
desoxypodophyllotoxin  
podophyllotoxin,  
deoxysilicicolin

19186-35-7

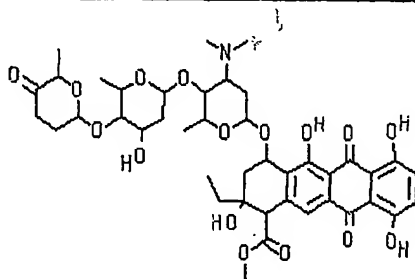
NCI 60-tumor panel  
(GI50's): ~100 nM to 3  
nM; (LC50): 50  $\mu$ M to  
10 nM



Scillaren A/  
not known/  
NSC-7525; Gluco-  
proscillaridin A;  
Scillaren A

124-99-2

NCI 60-tumor panel  
(GI50's): 50 nM to 0.1  
nM;  
(LC50): 250  $\mu$ M to 0.1  
nM



Cinerubin A-HCl/  
not known/  
NSC-243022; Cinerubin  
A hydrochloride;  
CL 86-F2 HCl;  
CL-86-F2-hydrochloride

not reported

NCI 60-tumor panel  
(GI50's): 15 nM to 10  
pM; (LC50): 100  $\mu$ M  
to 6 nM

<sup>1</sup> WO-09739025; US-6025466

<sup>2</sup> EP-00626383 30 November 1994

<sup>3</sup> JP-10101676

<sup>4</sup> WO-09705162; WO-09717364 (dolastatin synthesis and analogs)

<sup>5</sup> Kosan licensed patent for Epothilone analogs from Sloan-Kettering; US 00185968

- <sup>6</sup> WO-09723211  
<sup>7</sup> WO-09723211  
<sup>8</sup> JP-08092232  
<sup>9</sup> WO-09633211  
5 <sup>10</sup> EP-00608111; EP-00632042; EP-00634414; WO-09748278  
<sup>11</sup> EP-00425235; JP-53124692  
<sup>12</sup> US-05416064; US-05208020; EP-00425235B  
<sup>13</sup> EP-00425235I JP-53124692; US-06333410B1  
<sup>14</sup> JP-1104183  
10 <sup>15</sup> EP-00689845  
<sup>16</sup> EP-00689845  
<sup>17</sup> EP-00136791; EP-00087957  
<sup>18</sup> US 50001112; US 5143906.  
<sup>19</sup> WO-00136048  
15

Conventional immunotoxins contain an MAb chemically conjugated to a toxin that is mutated or chemically modified to minimized binding to normal cells. Examples include anti-B4-blocked ricin, targeting CD5; and RFB4-deglycosylated ricin A chain, targeting CD22. Recombinant immunotoxins developed more recently are chimeric proteins consisting of the variable region of an antibody directed against a tumor antigen fused to a protein toxin using recombinant DNA technology. The toxin is also frequently genetically modified to remove normal tissue binding sites but retain its cytotoxicity. A large number of differentiation antigens, overexpressed receptors, or cancer-specific antigens have been identified as targets for immunotoxins, *e.g.*, CD19, CD22, CD20, IL-2 receptor (CD25), CD33, IL-4 receptor, EGF receptor and its mutants, ErbB2, Lewis carbohydrate, mesothelin, transferrin receptor, GM-CSF receptor, Ras, Bcr-Abl, and c-Kit, for the treatment of a variety of malignancies including hematopoietic cancers, glioma, and breast, colon, ovarian, bladder, and gastrointestinal cancers. *See e.g.*, Brinkmann et al., *Expert Opin. Biol. Ther.* 1:693-702 (2001); Perentesis and Sievers, *Hematology/Oncology Clinics of North America* 15:677-701 (2001).

MAbs conjugated with radioisotope are used as another means of treating human malignancies, particularly hematopoietic malignancies, with a high level of specificity and effectiveness. The most commonly used isotopes for therapy are the high-energy  $\gamma$ -emitters, such as <sup>131</sup>I and <sup>90</sup>Y. Recently, <sup>213</sup>Bi-labeled anti-CD33 humanized MAb has also been tested in phase I human clinical trials. Reff et al., *supra*.

A number of MAbs have been used for therapeutic purposes. For example, the use of rituximab (Rituxan<sup>TM</sup>), a recombinant chimeric anti-CD20 MAb, for treating certain

hematopoietic malignancies was approved by the FDA in 1997. Other MABs that have since been approved for therapeutic uses in treating human cancers include: alemtuzumab (Campath-1H™), a humanized rat antibody against CD52; and gemtuzumab ozogamicin (Mylotarg™), a calicheamicin-conjugated humanized mouse antiCD33 MAB. The FDA is also currently examining the safety and efficacy of several other MABs for the purpose of site-specific delivery of cytotoxic agents or radiation, e.g., radiolabeled Zevalin™ and Bexxar™. Reff et al., *supra*.

A second important consideration in designing a drug delivery system is the accessibility of a target tissue to a therapeutic agent. This is an issue of particular concern in the case of treating a disease of the central nervous system (CNS), where the blood-brain barrier prevents the diffusion of macromolecules. Several approaches have been developed to bypass the blood-brain barrier for effective delivery of therapeutic agents to the CNS.

The understanding of iron transport mechanism from plasma to brain provides a useful tool in bypassing the blood-brain barrier (BBB). Iron, transported in plasma by transferrin, is an essential component of virtually all types of cells. The brain needs iron for metabolic processes and receives iron through transferrin receptors located on brain capillary endothelial cells via receptor-mediated transcytosis and endocytosis. Moos and Morgan, *Cellular and Molecular Neurobiology* 20:77-95 (2000). Delivery systems based on transferrin-transferrin receptor interaction have been established for the efficient delivery of peptides, proteins, and liposomes into the brain. For example, peptides can be coupled with a Mab directed against the transferrin receptor to achieve greater uptake by the brain, Moos and Morgan, *Supra*. Similarly, when coupled with an MAB directed against the transferrin receptor, the transportation of basic fibroblast growth factor (bFGF) across the blood-brain barrier is enhanced. Song et al., *The Journal of Pharmacology and Experimental Therapeutics* 301:605-610 (2002); Wu et al., *Journal of Drug Targeting* 10:239-245 (2002). In addition, a liposomal delivery system for effective transport of the chemotherapy drug, doxorubicin, into C6 glioma has been reported, where transferrin was attached to the distal ends of liposomal PEG chains. Eavarone et al., *J. Biomed. Mater. Res.* 51:10-14 (2000). A number of US patents also relate to delivery methods bypassing the blood-brain barrier based on transferrin-transferrin receptor interaction. See e.g., US Patent Nos. 5,154,924; 5,182,107; 5,527,527; 5,833,988; 6,015,555.

There are other suitable conjugation partners for a pharmaceutical agent to bypass the blood-brain barrier. For example, US Patent Nos. 5,672,683, 5,977,307 and WO 95/02421 relate to a method of delivering a neuropharmaceutical agent across the blood-brain barrier, where the agent is administered in the form of a fusion protein with a ligand that is reactive with a brain capillary endothelial cell receptor; WO 99/00150 describes a drug delivery system in which the transportation of a drug across the blood-brain barrier is facilitated by conjugation with an MAb directed against human insulin receptor; WO 89/10134 describes a chimeric peptide, which includes a peptide capable of crossing the blood brain barrier at a relatively high rate and a hydrophilic neuropeptide incapable of transcytosis, as a means of introducing hydrophilic neuropeptides into the brain; WO 01/60411 A1 provides a pharmaceutical composition that can easily transport a pharmaceutically active ingredient into the brain. The active ingredient is bound to a hibernation-specific protein that is used as a conjugate, and administered with a thyroid hormone or a substance promoting thyroid hormone production. In addition, an alternative route of drug delivery for bypassing the blood-brain barrier has been explored. For instance, intranasal delivery of therapeutic agents without the need for conjugation has been shown to be a promising alternative delivery method (Frey, 2002, *Drug Delivery Technology*, 2(5):46-49).

In addition to facilitating the transportation of drugs across the blood-brain barrier, transferrin-transferrin receptor interaction is also useful for specific targeting of certain tumor cells, as many tumor cells overexpress transferrin receptor on their surface. This strategy has been used for delivering bioactive macromolecules into K562 cells via a transferrin conjugate (Wellhoner et al., *The Journal of Biological Chemistry* 266:4309-4314 (1991)), and for delivering insulin into enterocyte-like Caco-2 cells via a transferrin conjugate (Shah and Shen, *Journal of Pharmaceutical Sciences* 85:1306-1311 (1996)).

Furthermore, as more becomes known about the functions of various iron transport proteins, such as lactotransferrin receptor, melanotransferrin, ceruloplasmin, and Divalent Cation Transporter and their expression pattern, some of the proteins involved in iron transport mechanism(e.g., melanotransferrin), or their fragments, have been found to be similarly effective in assisting therapeutic agents transport across the blood-brain barrier or targeting specific tissues (WO 02/13843 A2, WO 02/13873 A2). For a review on the use of

transferrin and related proteins involved in iron uptake as conjugates in drug delivery, *see* Li and Qian, *Medical Research Reviews* 22:225-250 (2002).

The concept of tissue-specific delivery of therapeutic agents goes beyond the interaction between transferrin and transferrin receptor or their related proteins. For example, a bone-specific delivery system has been described in which proteins are conjugated with a bone-seeking aminobisphosphate for improved delivery of proteins to mineralized tissue. Uludag and Yang, *Biotechnol. Prog.* 18:604-611 (2002). For a review on this topic, *see* Vyas et al., *Critical Reviews in Therapeutic Drug Carrier System* 18:1-76 (2001).

A variety of linkers may be used in the process of generating bioconjugates for the purpose of specific delivery of therapeutic agents. Suitable linkers include homo- and heterobifunctional cross-linking reagents, which may be cleavable by, e.g., acid-catalyzed dissociation, or non-cleavable (*see, e.g.*, Srinivasachar and Neville, *Biochemistry* 28:2501-2509 (1989); Wellhoner et al., *The Journal of Biological Chemistry* 266:4309-4314 (1991)). Interaction between many known binding partners, such as biotin and avidin/streptavidin, can also be used as a means to join a therapeutic agent and a conjugate partner that ensures the specific and effective delivery of the therapeutic agent. Using the methods of the invention, proteins may be used to deliver molecules to intracellular compartments as conjugates. Proteins, peptides, hormones, cytokines, small molecules or the like that bind to specific cell surface receptors that are internalized after ligand binding may be used for intracellular targeting of conjugated therapeutic compounds. Typically, the receptor-ligand complex is internalized into intracellular vesicles that are delivered to specific cell compartments, including, but not limited to, the nucleus, mitochondria, golgi, ER, lysosome, and endosome, depending on the intracellular location targeted by the receptor. By conjugating the receptor ligand with the desired molecule, the drug will be carried with the receptor-ligand complex and be delivered to the intracellular compartments normally targeted by the receptor. The drug can therefore be delivered to a specific intracellular location in the cell where it is needed to treat a disease.

Many proteins may be used to target therapeutic agents to specific tissues and organs. Targeting proteins include, but are not limited to, growth factors (EPO, HGH, EGF, nerve growth factor, FGF, among others), cytokines (GM-CSF, G-CSF, the interferon family, interleukins, among others), hormones (FSH, LH, the steroid families, estrogen,

corticosteroids, insulin, among others), serum proteins (albumin, lipoproteins, fetoprotein, human serum proteins, antibodies and fragments of antibodies, among others), and vitamins (folate, vitamin C, vitamin A, among others). Targeting agents are available that are specific for receptors on most cells types.

5        Contemplated linkage configurations include, but are not limited to, protein-sugar-linker-sugar-protein and multivalent forms thereof, protein-sugar-linker-protein and multivalent forms thereof, protein-sugar-linker-therapeutic agent, where the therapeutic agent includes, but are not limited to, small molecules, peptides and lipids. In some embodiments, a hydrolysable linker is used that can be hydrolyzed once internalized. An acid labile linker  
10       can be used to advantage where the protein conjugate is internalized into the endosomes or lysosomes which have an acidic pH. Once internalized into the endosome or lysosome, the linker is hydrolyzed and the therapeutic agent is released from the targeting agent.

      In an exemplary embodiment, transferrin is conjugated via a linker to an enzyme or a nucleic acid vector that encoded the enzyme desired to be targeted to a cell that presents  
15       transferrin receptors in a patient. The patient could, for example, require enzyme replacement therapy for that particular enzyme. In particularly preferred embodiments, the enzyme is one that is lacking in a patient with a lysosomal storage disease (see Table 5). Once in circulation, the transferrin-enzyme conjugate is linked to transferrin receptors and is internalized in early endosomes (Xing et al., 1998, Biochem. J. 336:667; Li et al., 2002,  
20       Trends in Pharmacol. Sci. 23:206; Suhaila et al., 1998, J. Biol. Chem. 273:14355). Other contemplated targeting agents that are related to transferrin include, but are not limited to, lactotransferrin (lactoferrin), melanotransferrin (p97), ceruloplasmin, and divalent cation transporter.

      In another exemplary embodiment, transferrin-dystrophin conjugates would enter  
25       endosomes by the transferrin pathway. Once there, the dystrophin is released due to a hydrolysable linker which can then be taken to the intracellular compartment where it is required. This embodiment may be used to treat a patient with muscular dystrophy by supplementing a genetically defective dystrophin gene and/or protein with the functional dystrophin peptide connected to the transferrin.

### E. Therapeutic Moieties

In another preferred embodiment, the modified sugar includes a therapeutic moiety. Those of skill in the art will appreciate that there is overlap between the category of therapeutic moieties and biomolecules; many biomolecules have therapeutic properties or potential.

The therapeutic moieties can be agents already accepted for clinical use or they can be drugs whose use is experimental, or whose activity or mechanism of action is under investigation. The therapeutic moieties can have a proven action in a given disease state or can be only hypothesized to show desirable action in a given disease state. In a preferred embodiment, the therapeutic moieties are compounds, which are being screened for their ability to interact with a tissue of choice. Therapeutic moieties, which are useful in practicing the instant invention include drugs from a broad range of drug classes having a variety of pharmacological activities. In some embodiments, it is preferred to use therapeutic moieties that are not sugars. An exception to this preference is the use of a sugar that is modified by covalent attachment of another entity, such as a PEG, biomolecule, therapeutic moiety, diagnostic moiety and the like. In an exemplary embodiment, an antisense nucleic acid moiety is conjugated to a linker arm which is attached to the targeting moiety. In another exemplary embodiment, a therapeutic sugar moiety is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

Methods of conjugating therapeutic and diagnostic agents to various other species are well known to those of skill in the art. See, for example Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.

In an exemplary embodiment, the therapeutic moiety is attached to the modified sugar via a linkage that is cleaved under selected conditions. Exemplary conditions include, but are not limited to, a selected pH (e.g., stomach, intestine, endocytotic vacuole), the presence of an active enzyme (e.g., esterase, protease, reductase, oxidase), light, heat and the like. Many cleavable groups are known in the art. See, for example, Jung *et al.*, *Biochem. Biophys. Acta*, 761: 152-162 (1983); Joshi *et al.*, *J. Biol. Chem.*, 265: 14518-14525 (1990); Zarling *et al.*, *J.*



*Immunol.*, 124: 913-920 (1980); Bouiziar *et al.*, *Eur. J. Biochem.*, 155: 141-147 (1986); Park *et al.*, *J. Biol. Chem.*, 261: 205-210 (1986); Browning *et al.*, *J. Immunol.*, 143: 1859-1867 (1989).

- Classes of useful therapeutic moieties include, for example, non-steroidal anti-inflammatory drugs (NSAIDS). The NSAIDS can, for example, be selected from the following categories: (e.g., propionic acid derivatives, acetic acid derivatives, fenamic acid derivatives, biphenylcarboxylic acid derivatives and oxicams); steroidal anti-inflammatory drugs including hydrocortisone and the like; adjuvants; antihistaminic drugs (e.g., chlorpheniramine, triprolidine); antitussive drugs (e.g., dextromethorphan, codeine, caramiphen and carbetapentane); antipruritic drugs (e.g., methdilazine and trimeprazine); anticholinergic drugs (e.g., scopolamine, atropine, homatropine, levodopa); anti-emetic and antinauseant drugs (e.g., cyclizine, meclizine, chlorpromazine, buclizine); anorexic drugs (e.g., benzphetamine, phentermine, chlorphentermine, fenfluramine); central stimulant drugs (e.g., amphetamine, methamphetamine, dextroamphetamine and methylphenidate); antiarrhythmic drugs (e.g., propranolol, procainamide, disopyramide, quinidine, encainide);  $\beta$ -adrenergic blocker drugs (e.g., metoprolol, acebutolol, betaxolol, labetalol and timolol); cardiotonic drugs (e.g., milrinone, amrinone and dobutamine); antihypertensive drugs (e.g., enalapril, clonidine, hydralazine, minoxidil, guanadrel, guanethidine); diuretic drugs (e.g., amiloride and hydrochlorothiazide); vasodilator drugs (e.g., diltiazem, amiodarone, isoxsuprine, nylidrin, tolazoline and verapamil); vasoconstrictor drugs (e.g., dihydroergotamine, ergotamine and methylsergide); antiulcer drugs (e.g., ranitidine and cimetidine); anesthetic drugs (e.g., lidocaine, bupivacaine, chlorprocaine, dibucaine); antidepressant drugs (e.g., imipramine, desipramine, amitriptyline, nortriptyline); tranquilizer and sedative drugs (e.g., chlordiazepoxide, benactyzine, benzquinamide, flurazepam, hydroxyzine, loxapine and promazine); antipsychotic drugs (e.g., chlorprothixene, fluphenazine, haloperidol, molindone, thioridazine and trifluoperazine); antimicrobial drugs (antibacterial, antifungal, antiprotozoal and antiviral drugs).

- Classes of useful therapeutic moieties include adjuvants. The adjuvants can, for example, be selected from keyhole lymphet hemocyanin conjugates, monophosphoryl lipid A, mycoplasma-derived lipopeptide MALP-2, cholera toxin B subunit, *Escherichia coli* heat-labile toxin, universal T helper epitope from tetanus toxoid, interleukin-12, CpG

oligodeoxynucleotides, dimethyldioctadecylammonium bromide, cyclodextrin, squalene, aluminum salts, meningococcal outer membrane vesicle (OMV), montanide ISA, TiterMax™ (available from Sigma, St. Louis MO), nitrocellulose absorption, immune-stimulating complexes such as Quil A, Gerbu™ adjuvant (Gerbu Biotechnik, Kirchwald, Germany),  
5 threonyl muramyl dipeptide, thymosin alpha, bupivacaine, GM-CSF, Incomplete Freund's Adjuvant, MTP-PE/MF59 (Ciba/Geigy, Basel, Switzerland), polyphosphazene, saponin derived from the soapbark tree *Quillaja saponaria*, and Syntex adjuvant formulation (Biocine, Emeryville, CA), among others well known to those in the art.

Antimicrobial drugs which are preferred for incorporation into the present  
10 composition include, for example, pharmaceutically acceptable salts of  $\beta$ -lactam drugs, quinolone drugs, ciprofloxacin, norfloxacin, tetracycline, erythromycin, amikacin, triclosan, doxycycline, capreomycin, chlorhexidine, chlortetracycline, oxytetracycline, clindamycin, ethambutol, hexamidine isothionate, metronidazole, pentamidine, gentamycin, kanamycin, lineomycin, methacycline, methenamine, minocycline, neomycin, netilmycin, paromomycin,  
15 streptomycin, tobramycin, miconazole and amantadine.

Other drug moieties of use in practicing the present invention include antineoplastic drugs (e.g., antiandrogens (e.g., leuprolide or flutamide), cytocidal agents (e.g., adriamycin, doxorubicin, taxol, cyclophosphamide, busulfan, cisplatin,  $\beta$ -2-interferon) anti-estrogens (e.g., tamoxifen), antimetabolites (e.g., fluorouracil, methotrexate, mercaptopurine,  
20 thioguanine). Also included within this class are radioisotope-based agents for both diagnosis and therapy, and conjugated toxins, such as ricin, geldanamycin, mytansin, CC-1065, C-1027, the duocarmycins, calicheamycin and related structures and analogues thereof, and the toxins listed in Table 2.

The therapeutic moiety can also be a hormone (e.g., medroxyprogesterone, estradiol,  
25 leuprolide, megestrol, octreotide or somatostatin); muscle relaxant drugs (e.g., cinnamedrine, cyclobenzaprine, flavoxate, orphenadrine, papaverine, mebeverine, idaverine, ritodrine, diphenoxylate, dantrolene and azumolen); antispasmodic drugs; bone-active drugs (e.g., diphosphonate and phosphonoalkylphosphinate drug compounds); endocrine modulating drugs (e.g., contraceptives (e.g., ethinodiol, ethinyl estradiol, norethindrone, mestranol,  
30 desogestrel, medroxyprogesterone), modulators of diabetes (e.g., glyburide or chlorpropamide), anabolics, such as testolactone or stanozolol, androgens (e.g.,

methyltestosterone, testosterone or fluoxymesterone), antidiuretics (*e.g.*, desmopressin) and calcitonins).

Also of use in the present invention are estrogens (*e.g.*, diethylstilbesterol), glucocorticoids (*e.g.*, triamcinolone, betamethasone, etc.) and progesterones, such as norethindrone, ethynodiol, norethindrone, levonorgestrel; thyroid agents (*e.g.*, liothyronine or levothyroxine) or anti-thyroid agents (*e.g.*, methimazole); antihyperprolactinemic drugs (*e.g.*, cabergoline); hormone suppressors (*e.g.*, danazol or goserelin), oxytocics (*e.g.*, methylegonovine or oxytocin) and prostaglandins, such as mioprostol, alprostadil or dinoprostone, can also be employed.

Other useful modifying groups include immunomodulating drugs (*e.g.*, antihistamines, mast cell stabilizers, such as lodoxamide and/or cromolyn, steroids (*e.g.*, triamcinolone, beclomethazone, cortisone, dexamethasone, prednisolone, methylprednisolone, beclomethasone, or clobetasol), histamine H<sub>2</sub> antagonists (*e.g.*, famotidine, cimetidine, ranitidine), immunosuppressants (*e.g.*, azathioprine, cyclosporin), etc. Groups with anti-inflammatory activity, such as sulindac, etodolac, ketoprofen and ketorolac, are also of use. Other drugs of use in conjunction with the present invention will be apparent to those of skill in the art.

Classes of useful therapeutic moieties include, for example, antisense drugs and also naked DNA. The antisense drugs can be selected from for example Affinitak (ISIS, Carlsbad, CA) and Genasense<sup>™</sup> (from Genta, Berkeley Heights, NJ). Naked DNA can be delivered as a gene therapy therapeutic for example with the DNA encoding for example factors VIII and IX for treatment of hemophilia disorders.

#### F. Preparation of Modified Sugars

Modified sugars useful in forming the conjugates of the invention are discussed herein. The discussion focuses on preparing a sugar modified with a water-soluble polymer for clarity of illustration. In particular, the discussion focuses on the preparation of modified sugars that include a poly(ethylene glycol) moiety. Those of skill will appreciate that the methods set forth herein are broadly applicable to the preparation of modified sugars, therefore, the discussion should not be interpreted as limiting the scope of the invention.

In general, the sugar moiety and the modifying group are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or unreactive species. The sugar reactive functional group(s), is located at any position on the sugar moiety. Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. Currently favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, Smith and March, *ADVANCED ORGANIC CHEMISTRY*, 5th Ed., John Wiley & Sons, New York, 2001; Hermanson, *BIOCONJUGATE TECHNIQUES*, Academic Press, San Diego, 1996; and Feeney *et al.*, *MODIFICATION OF PROTEINS*; Advances in Chemistry Series, Vol. 198, American Chemical Society, Washington, D.C., 1982.

Useful reactive functional groups pendent from a sugar nucleus or modifying group include, but are not limited to:

(a) carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenzotriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;

(b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*

(c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional group of the halogen atom;

(d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition;

(f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with alkyl and acyl halides;

5 (h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc*; and

(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

10 The reactive functional groups can be chosen such that they do not participate in, or interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of  
15 reaction conditions. For examples of useful protecting groups, *see*, for example, Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

In the discussion that follows, a number of specific examples of modified sugars that are useful in practicing the present invention are set forth. In the exemplary embodiments, a sialic acid derivative is utilized as the sugar nucleus to which the modifying group is  
20 attached. The focus of the discussion on sialic acid derivatives is for clarity of illustration only and should not be construed to limit the scope of the invention. Those of skill in the art will appreciate that a variety of other sugar moieties can be activated and derivatized in a manner analogous to that set forth using sialic acid as an example. For example, numerous methods are available for modifying galactose, glucose, N-acetylgalactosamine and fucose to  
25 name a few sugar substrates, which are readily modified by art recognized methods. *See*, for example, Elhalabi *et al.*, *Curr. Med. Chem.* 6: 93 (1999); and Schafer *et al.*, *J. Org. Chem.* 65: 24 (2000).

In an exemplary embodiment, the peptide that is modified by a method of the invention is a peptide that is produced in mammalian cells (e.g., CHO cells) or in a transgenic  
30 animal and thus, contains N- and/or O-linked oligosaccharide chains, which are incompletely sialylated. The oligosaccharide chains of the glycopeptide lacking a sialic acid and

containing a terminal galactose residue can be PEGylated, PPGylated or otherwise modified with a modified sialic acid.

In Scheme 4, the mannosamine glycoside **1**, is treated with the active ester of a protected amino acid (*e.g.*, glycine) derivative, converting the sugar amine residue into the corresponding protected amino acid amide adduct. The adduct is treated with an aldolase to form the sialic acid **2**. Compound **2** is converted to the corresponding CMP derivative by the action of CMP-SA synthetase, followed by catalytic hydrogenation of the CMP derivative to produce compound **3**. The amine introduced via formation of the glycine adduct is utilized as a locus of PEG or PPG attachment by reacting compound **3** with an activated PEG or PPG derivative (*e.g.*, PEG-C(O)NHS, PPG-C(O)NHS), producing **4** or **5**, respectively.

Scheme 4

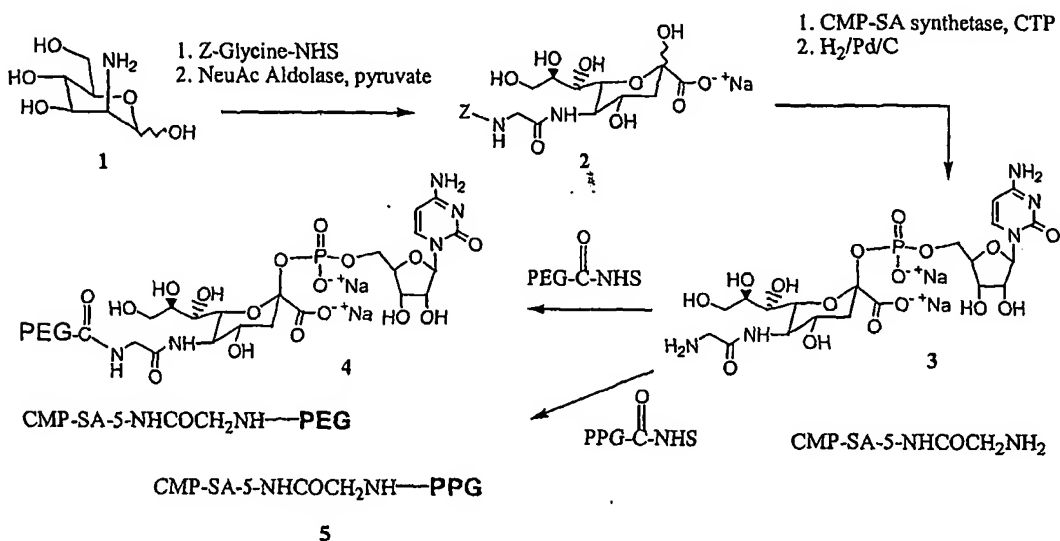
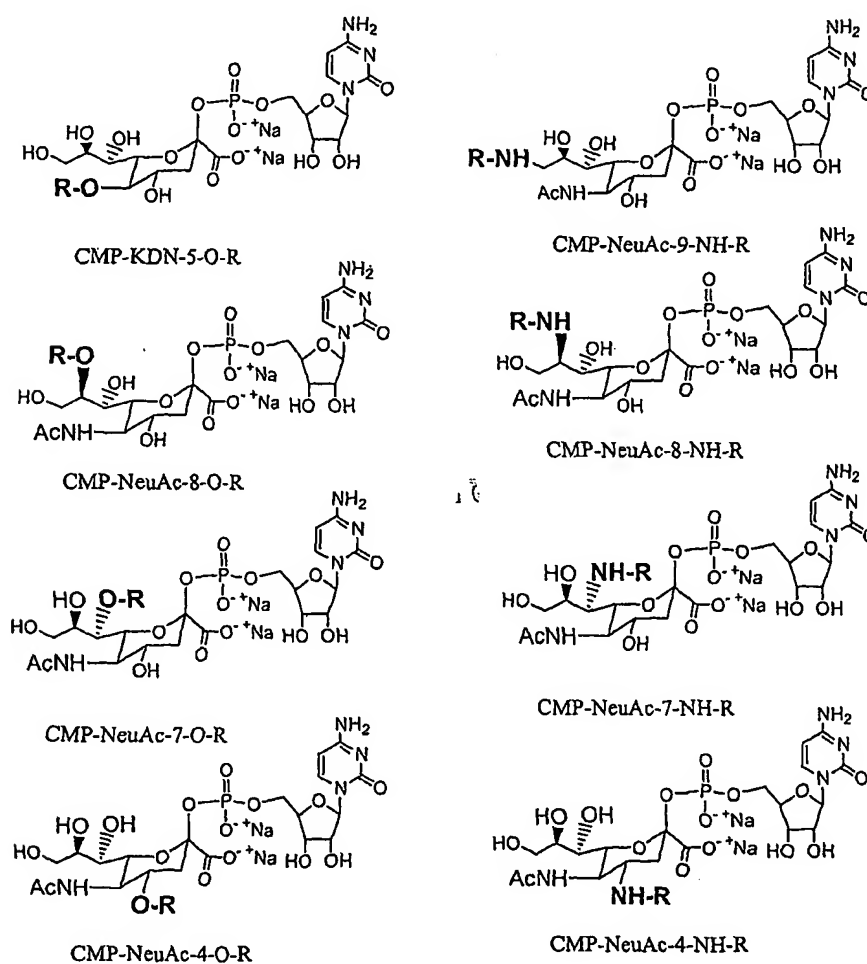
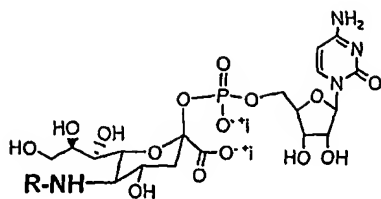


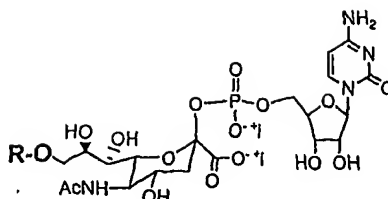
Table 3 sets forth representative examples of sugar monophosphates that are derivatized with a PEG or PPG moiety. Certain of the compounds of Table 3 are prepared by the method of Scheme 1. Other derivatives are prepared by art-recognized methods. See, for example, Keppler *et al.*, *Glycobiology* 11: 11R (2001); and Charter *et al.*, *Glycobiology* 10: 1049 (2000)). Other amine reactive PEG and PPG analogues are commercially available, or they can be prepared by methods readily accessible to those of skill in the art.

**Table 3.** Examples of sugar monophosphates that are derivatized with a PEG or PPG moiety



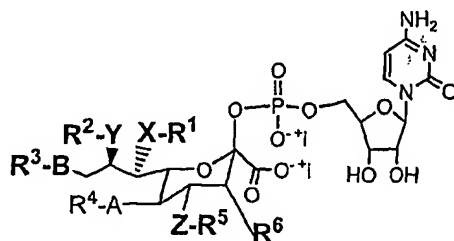


CMP-SA-5-NH-R



CMP-NeuAc-9-O-R

The modified sugar phosphates of use in practicing the present invention can be substituted in other positions as well as those set forth above. "i" may be Na or another salt and "i" may be interchangeable with Na. Presently preferred substitutions of sialic acid are set forth in Formula 5.

**Formula 5:**

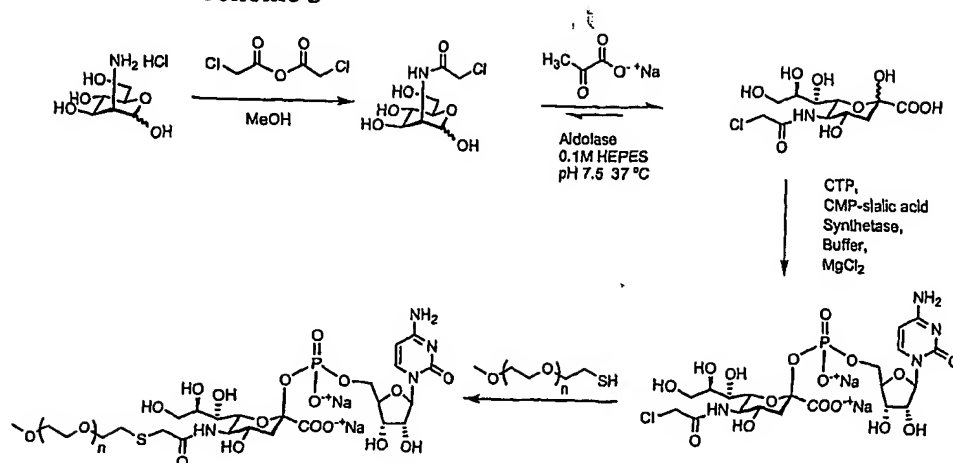
(I)

in which X is a linking group, which is preferably selected from  $-O-$ ,  $-N(H)-$ ,  $-S-$ ,  $CH_2-$ , and  $N(R)_2$ , in which each R is a member independently selected from  $R^1$ - $R^5$ . "i" may be Na or another salt, and Na may be interchangeable with "i". The symbols Y, Z, A and B each represent a group that is selected from the group set forth above for the identity of X. X, Y, Z, A and B are each independently selected and, therefore, they can be the same or different. The symbols  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  represent H, polymers, a water-soluble polymer, therapeutic moiety, biomolecule or other moiety. The symbol  $R^6$  represents H, OH, or a polymer. Alternatively, these symbols represent a linker that is linked to a polymer, water-soluble polymer, therapeutic moiety, biomolecule or other moiety.

In another exemplary embodiment, a mannosamine is simultaneously acylated and activated for a nucleophilic substitution by the use of chloroacetic anhydride as set forth in Scheme 5. In each of the schemes presented in this section,  $i^+$  or  $Na^+$  can be interchangeable, wherein the salt can be sodium, or can be any other suitable salt.



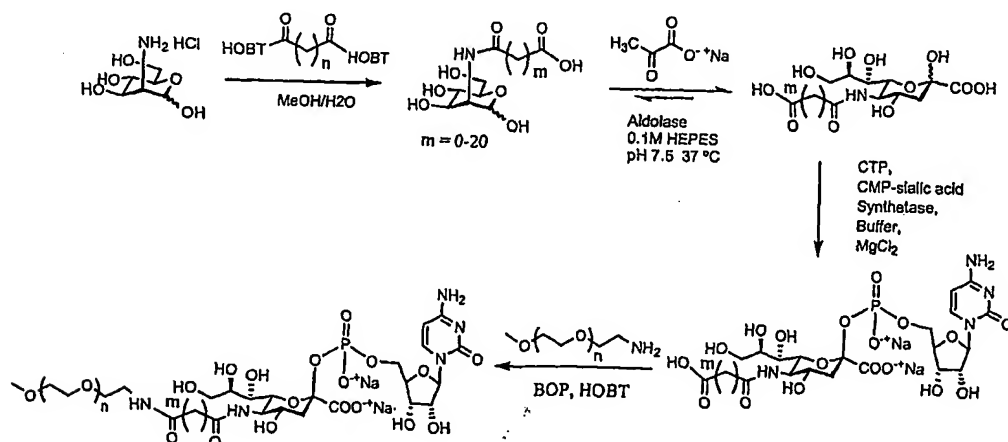
Scheme 5



The resulting chloro-derivatized glycan is contacted with pyruvate in the presence of an aldolase, forming a chloro-derivatized sialic acid. The corresponding nucleotide sugar is prepared by contacting the sialic acid derivative with an appropriate nucleotide triphosphates and a synthetase. The chloro group on the sialic acid moiety is then displaced with a nucleophilic PEG derivative, such as thio-PEG.

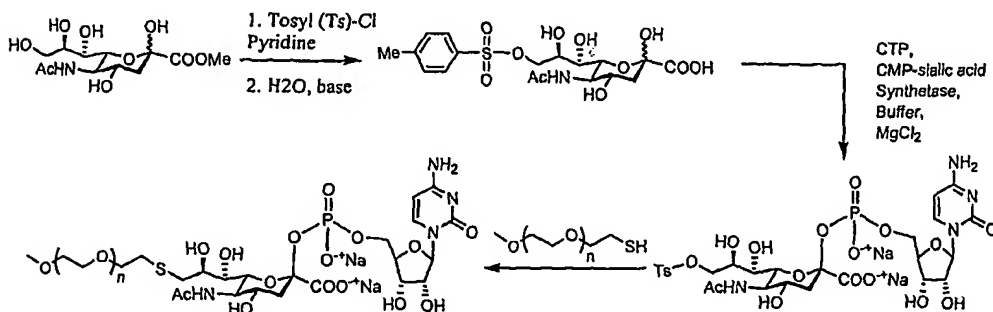
In a further exemplary embodiment, as shown in Scheme 6, a mannosamine is acylated with a bis-HOBT dicarboxylate, producing the corresponding amido-alkyl-carboxylic acid, which is subsequently converted to a sialic acid derivative. The sialic acid derivative is converted to a nucleotide sugar, and the carboxylic acid is activated and reacted with a nucleophilic PEG derivative, such as amino-PEG.

Scheme 6



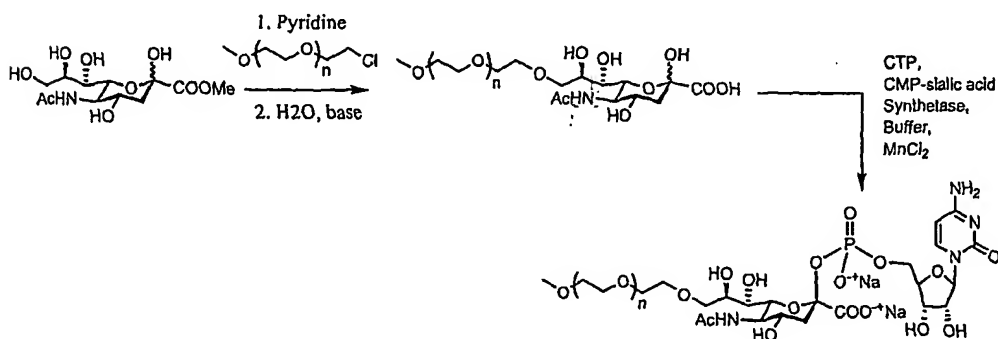
In another exemplary embodiment, set forth in Scheme 7, amine- and carboxyl-protected neuraminic acid is activated by converting the primary hydroxyl group to the corresponding p-toluenesulfonate ester, and the methyl ester is cleaved. The activated neuraminic acid is converted to the corresponding nucleotide sugar, and the activating group is displaced by a nucleophilic PEG species, such as thio-PEG.

Scheme 7



In yet a further exemplary embodiment, as set forth in Scheme 8, the primary hydroxyl moiety of an amine- and carboxyl-protected neuraminic acid derivative is alkylated using an electrophilic PEG, such as chloro-PEG. The methyl ester is subsequently cleaved and the PEG-sugar is converted to a nucleotide sugar.

Scheme 8

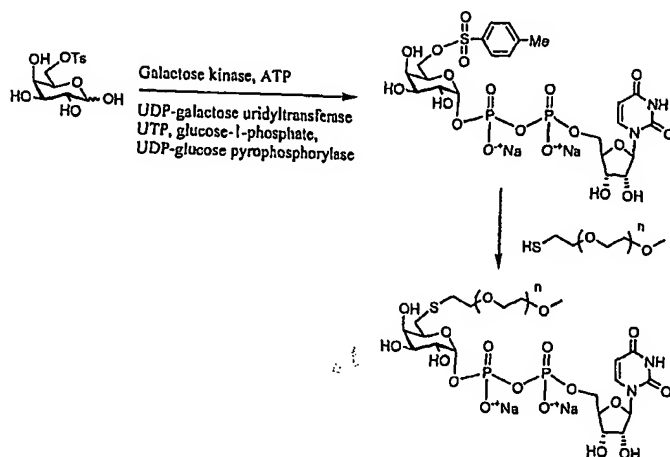


Glycans other than sialic acid can be derivatized with PEG using the methods set forth herein. The derivatized glycans, themselves, are also within the scope of the invention.

Thus, Scheme 9 provides an exemplary synthetic route to a PEGylated galactose nucleotide

sugar. The primary hydroxyl group of galactose is activated as the corresponding toluenesulfonate ester, which is subsequently converted to a nucleotide sugar.

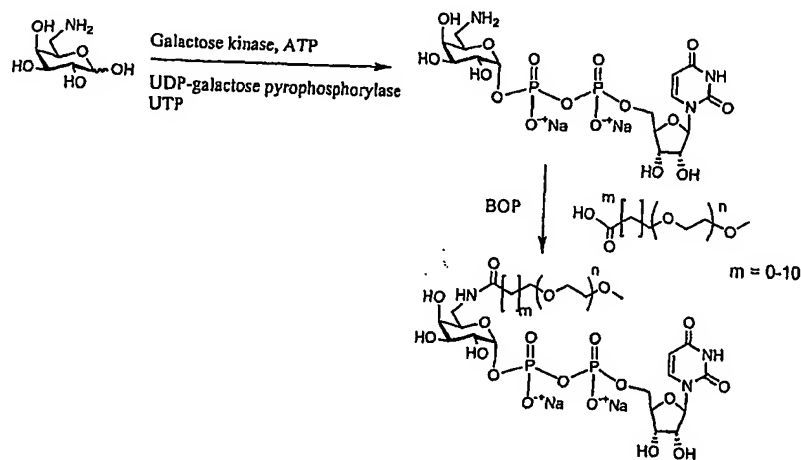
Scheme 9



5

Scheme 10 sets forth an exemplary route for preparing a galactose-PEG derivative that is based upon a galactose-6-amine moiety. Thus, galactosamine is converted to a nucleotide sugar, and the amine moiety of galactosamine is functionalized with an active PEG derivative.

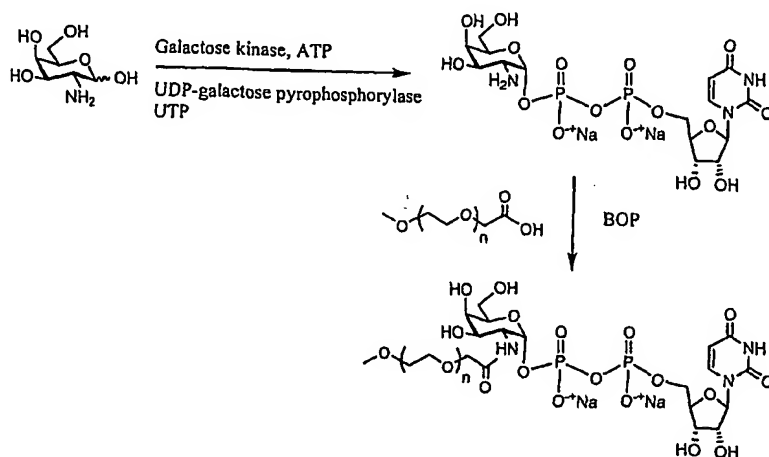
Scheme 10



Scheme 11 provides another exemplary route to galactose derivatives. The starting point for Scheme 11 is galactose-2-amine, which is converted to a nucleotide sugar. The amine moiety of the nucleotide sugar is the locus for attaching a PEG derivative, such as Methoxy-PEG (mPEG) carboxylic acid.

5

Scheme 11



Exemplary moieties attached to the conjugates disclosed herein include, but are not limited to, PEG derivatives (e.g., acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG, alkyl-PEG), PPG derivatives (e.g., acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG), polyapartac acid, polyglutamate, polylysine, therapeutic moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, SLe<sup>x</sup>, mannose, mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins (e.g., transferrin), chondroitin, keratan, dermatan, dextran, modified dextran, amylose, bisphosphate, poly-SA, hyaluronic acid, keritan, albumin, integrins, antennary oligosaccharides, peptides and the like. Methods of conjugating the various modifying groups to a saccharide moiety are readily accessible to those of skill in the art (POLY (ETHYLENE GLYCOL CHEMISTRY : BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, J. Milton Harris, Ed., Plenum Pub. Corp., 1992; POLY (ETHYLENE GLYCOL) CHEMICAL AND BIOLOGICAL APPLICATIONS, J. Milton Harris, Ed., ACS Symposium Series No. 680, American Chemical Society, 1997; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS

AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Purification of sugars, nucleotide sugars and derivatives

The nucleotide sugars and derivatives produced by the above processes can be used without purification. However, it is usually preferred to recover the product. Standard, well-known techniques for recovery of glycosylated saccharides such as thin or thick layer chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the recovery as is discussed hereinafter and in the literature cited herein. For instance, membrane filtration wherein the membranes have molecular weight cutoff of about 3000 to about 10,000 can be used to remove proteins for reagents having a molecular weight of less than 10,000 Da.. Membrane filtration or reverse osmosis can then be used to remove salts and/or purify the product saccharides (*see, e.g.*, WO 98/15581). Nanofilter membranes are a class of reverse osmosis membranes that pass monovalent salts but retain polyvalent salts and uncharged solutes larger than about 100 to about 2,000 Daltons, depending upon the membrane used. Thus, in a typical application, saccharides prepared by the methods of the present invention will be retained in the membrane and contaminating salts will pass through.

G. Cross-linking Groups

Preparation of the modified sugar for use in the methods of the present invention includes attachment of a modifying group to a sugar residue and forming a stable adduct, which is a substrate for a glycosyltransferase. Thus, it is often preferred to use a cross-linking agent to conjugate the modifying group and the sugar. Exemplary bifunctional compounds which can be used for attaching modifying groups to carbohydrate moieties include, but are not limited to, bifunctional poly(ethylene glycols), polyamides, polyethers, polyesters and the like. General approaches for linking carbohydrates to other molecules are known in the literature. *See, for example*, Lee *et al.*, *Biochemistry* 28: 1856 (1989); Bhatia *et al.*, *Anal. Biochem.* 178: 408 (1989); Janda *et al.*, *J. Am. Chem. Soc.* 112: 8886 (1990) and Bednarski *et al.*, WO 92/18135. In the discussion that follows, the reactive groups are treated as benign on the sugar moiety of the nascent modified sugar. The focus of the discussion is

for clarity of illustration. Those of skill in the art will appreciate that the discussion is relevant to reactive groups on the modifying group as well.

An exemplary strategy involves incorporation of a protected sulfhydryl onto the sugar using the heterobifunctional crosslinker SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate and then deprotecting the sulfhydryl for formation of a disulfide bond with another sulfhydryl on the modifying group.

If SPDP detrimentally affects the ability of the modified sugar to act as a glycosyltransferase substrate, one of an array of other crosslinkers such as 2-iminothiolane or N-succinimidyl S-acetylthioacetate (SATA) is used to form a disulfide bond. 2-iminothiolane reacts with primary amines, instantly incorporating an unprotected sulfhydryl onto the amine-containing molecule. SATA also reacts with primary amines, but incorporates a protected sulfhydryl, which is later deacetylated using hydroxylamine to produce a free sulfhydryl. In each case, the incorporated sulfhydryl is free to react with other sulfhydryls or protected sulfhydryl, like SPDP, forming the required disulfide bond.

The above-described strategy is exemplary, and not limiting, of linkers of use in the invention. Other crosslinkers are available that can be used in different strategies for crosslinking the modifying group to the peptide. For example, TPCH(S-(2-thiopyridyl)-L-cysteine hydrazide and TPMPH ((S-(2-thiopyridyl) mercapto-propionohydrazide) react with carbohydrate moieties that have been previously oxidized by mild periodate treatment, thus forming a hydrazone bond between the hydrazide portion of the crosslinker and the periodate generated aldehydes. TPCH and TPMPH introduce a 2-pyridylthione protected sulfhydryl group onto the sugar, which can be deprotected with DTT and then subsequently used for conjugation, such as forming disulfide bonds between components.

If disulfide bonding is found unsuitable for producing stable modified sugars, other crosslinkers may be used that incorporate more stable bonds between components. The heterobifunctional crosslinkers GMBS (N-gamma-malimidobutyryloxy)succinimide) and SMCC (succinimidyl 4-(N-maleimido-methyl)cyclohexane) react with primary amines, thus introducing a maleimide group onto the component. The maleimide group can subsequently react with sulfhydryls on the other component, which can be introduced by previously mentioned crosslinkers, thus forming a stable thioether bond between the components. If steric hindrance between components interferes with either component's activity or the ability

of the modified sugar to act as a glycosyltransferase substrate, crosslinkers can be used which introduce long spacer arms between components and include derivatives of some of the previously mentioned crosslinkers (*i.e.*, SPDP). Thus, there is an abundance of suitable crosslinkers, which are useful; each of which is selected depending on the effects it has on optimal peptide conjugate and modified sugar production.

A variety of reagents are used to modify the components of the modified sugar with intramolecular chemical crosslinks (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., *Meth. Enzymol.* 25: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcenberg, and Roberts, eds.) pp. 395-442, Wiley, New York, 1981; Ji, T. H., *Meth. Enzymol.* 91: 580-609, 1983; Mattson *et al.*, *Mol. Biol. Rep.* 17: 167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material. Agents that catalyze formation of a disulfide bond belong to this category. Another example is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide  $\gamma$ -glutamyltransferase; EC 2.3.2.13) may be used as zero-length crosslinking reagent. This enzyme catalyzes acyl transfer reactions at carboxamide groups of protein-linked glutaminyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulfhydryl, guanidino, indole, or nonspecific groups.

## 2. Preferred Specific Sites in Crosslinking Reagents

### a. Amino-Reactive Groups

In one preferred embodiment, the sites on the cross-linker are amino-reactive groups. Useful non-limiting examples of amino-reactive groups include N-hydroxysuccinimide (NHS) esters, imidoesters, isocyanates, acylhalides, arylazides, p-nitrophenyl esters, aldehydes, and sulfonyl chlorides.

NHS esters react preferentially with the primary (including aromatic) amino groups of a modified sugar component. The imidazole groups of histidines are known to compete with primary amines for reaction, but the reaction products are unstable and readily hydrolyzed. The reaction involves the nucleophilic attack of an amine on the acid carboxyl of an NHS ester to form an amide, releasing the N-hydroxysuccinimide. Thus, the positive charge of the original amino group is lost.

Imidoesters are the most specific acylating reagents for reaction with the amine groups of the modified sugar components. At a pH between 7 and 10, imidoesters react only with primary amines. Primary amines attack imidates nucleophilically to produce an intermediate that breaks down to amidine at high pH or to a new imidate at low pH. The new imidate can react with another primary amine, thus crosslinking two amino groups, a case of a putatively monofunctional imidate reacting bifunctionally. The principal product of reaction with primary amines is an amidine that is a stronger base than the original amine. The positive charge of the original amino group is therefore retained.

Isocyanates (and isothiocyanates) react with the primary amines of the modified sugar components to form stable bonds. Their reactions with sulfhydryl, imidazole, and tyrosyl groups give relatively unstable products.

Acylazides are also used as amino-specific reagents in which nucleophilic amines of the affinity component attack acidic carboxyl groups under slightly alkaline conditions, *e.g.* pH 8.5.

Arylhalides such as 1,5-difluoro-2,4-dinitrobenzene react preferentially with the amino groups and tyrosine phenolic groups of modified sugar components, but also with sulfhydryl and imidazole groups.

p-Nitrophenyl esters of mono- and dicarboxylic acids are also useful amino-reactive groups. Although the reagent specificity is not very high,  $\alpha$ - and  $\epsilon$ -amino groups appear to react most rapidly.

Aldehydes such as glutaraldehyde react with primary amines of modified sugar. Although unstable Schiff bases are formed upon reaction of the amino groups with the aldehydes of the aldehydes, glutaraldehyde is capable of modifying the modified sugar with stable crosslinks. At pH 6-8, the pH of typical crosslinking conditions, the cyclic polymers undergo a dehydration to form  $\alpha$ - $\beta$  unsaturated aldehyde polymers. Schiff bases, however,



are stable, when conjugated to another double bond. The resonant interaction of both double bonds prevents hydrolysis of the Schiff linkage. Furthermore, amines at high local concentrations can attack the ethylenic double bond to form a stable Michael addition product.

5 Aromatic sulfonyl chlorides react with a variety of sites of the modified sugar components, but reaction with the amino groups is the most important, resulting in a stable sulfonamide linkage.

#### b. Sulfhydryl-Reactive Groups

10 In another preferred embodiment, the sites are sulfhydryl-reactive groups. Useful, non-limiting examples of sulfhydryl-reactive groups include maleimides, alkyl halides, pyridyl disulfides, and thiophthalimides.

Maleimides react preferentially with the sulfhydryl group of the modified sugar components to form stable thioether bonds. They also react at a much slower rate with  
15 primary amino groups and the imidazole groups of histidines. However, at pH 7 the maleimide group can be considered a sulfhydryl-specific group, since at this pH the reaction rate of simple thiols is 1000-fold greater than that of the corresponding amine.

Alkyl halides react with sulfhydryl groups, sulfides, imidazoles, and amino groups. At neutral to slightly alkaline pH, however, alkyl halides react primarily with sulfhydryl  
20 groups to form stable thioether bonds. At higher pH, reaction with amino groups is favored.

Pyridyl disulfides react with free sulfhydryls via disulfide exchange to give mixed disulfides. As a result, pyridyl disulfides are the most specific sulfhydryl-reactive groups.

Thiophthalimides react with free sulfhydryl groups to form disulfides.

#### c. Carboxyl-Reactive Residue

25 In another embodiment, carbodiimides soluble in both water and organic solvent, are used as carboxyl-reactive reagents. These compounds react with free carboxyl groups forming a pseudourea that can then be coupled to available amines yielding an amide linkage. Procedures to modify a carboxyl group with carbodiimide is well known in the art (see,  
30 Yamada *et al.*, *Biochemistry* 20: 4836-4842, 1981).

### 3. Preferred Nonspecific Sites in Crosslinking Reagents

In addition to the use of site-specific reactive moieties, the present invention contemplates the use of non-specific reactive groups to link the sugar to the modifying group.

Exemplary non-specific cross-linkers include photoactivatable groups, completely  
5 inert in the dark, which are converted to reactive species upon absorption of a photon of appropriate energy. In one preferred embodiment, photoactivatable groups are selected from precursors of nitrenes generated upon heating or photolysis of azides. Electron-deficient nitrenes are extremely reactive and can react with a variety of chemical bonds including N-H, O-H, C-H, and C=C. Although three types of azides (aryl, alkyl, and acyl derivatives) may  
10 be employed, arylazides are presently preferred. The reactivity of arylazides upon photolysis is better with N-H and O-H than C-H bonds. Electron-deficient aryl nitrenes rapidly ring-expand to form dehydroazepines, which tend to react with nucleophiles, rather than form C-H insertion products. The reactivity of arylazides can be increased by the presence of electron-withdrawing substituents such as nitro or hydroxyl groups in the ring. Such substituents push  
15 the absorption maximum of arylazides to longer wavelength. Unsubstituted arylazides have an absorption maximum in the range of 260-280 nm, while hydroxy and nitroarylazides absorb significant light beyond 305 nm. Therefore, hydroxy and nitroarylazides are most preferable since they allow to employ less harmful photolysis conditions for the affinity component than unsubstituted arylazides.

20 In another preferred embodiment, photoactivatable groups are selected from fluorinated arylazides. The photolysis products of fluorinated arylazides are aryl nitrenes, all of which undergo the characteristic reactions of this group, including C-H bond insertion, with high efficiency (Keana *et al.*, *J. Org. Chem.* **55**: 3640-3647, 1990).

In another embodiment, photoactivatable groups are selected from benzophenone  
25 residues. Benzophenone reagents generally give higher crosslinking yields than arylazide reagents.

In another embodiment, photoactivatable groups are selected from diazo compounds, which form an electron-deficient carbene upon photolysis. These carbenes undergo a variety of reactions including insertion into C-H bonds, addition to double bonds (including aromatic  
30 systems), hydrogen attraction and coordination to nucleophilic centers to give carbon ions.

In still another embodiment, photoactivatable groups are selected from diazopyruvates. For example, the p-nitrophenyl ester of p-nitrophenyl diazopyruvate reacts with aliphatic amines to give diazopyruvic acid amides that undergo ultraviolet photolysis to form aldehydes. The photolyzed diazopyruvate-modified affinity component will react like  
 5 formaldehyde or glutaraldehyde forming crosslinks.

#### 4. Homobifunctional Reagents

##### a. Homobifunctional crosslinkers reactive with primary amines

Synthesis, properties, and applications of amine-reactive cross-linkers are  
 10 commercially described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many reagents are available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR.).

Preferred, non-limiting examples of homobifunctional NHS esters include disuccinimidyl glutarate (DSG), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS), disuccinimidyl tartarate (DST), disulfosuccinimidyl tartarate (sulfo-DST), bis-  
 15 2-(succinimidooxycarbonyloxy)ethylsulfone (BSOCOES), bis-2-(sulfosuccinimidooxycarbonyloxy)ethylsulfone (sulfo-BSOCOES), ethylene glycolbis(succinimidylsuccinate) (EGS), ethylene glycolbis(sulfosuccinimidylsuccinate) (sulfo-EGS), dithiobis(succinimidylpropionate) (DSP), and dithiobis(sulfosuccinimidylpropionate) (sulfo-DSP). Preferred, non-  
 20 limiting examples of homobifunctional imidoesters include dimethyl malonimide (DMM), dimethyl succinimide (DMSC), dimethyl adipimide (DMA), dimethyl pimelimide (DMP), dimethyl suberimide (DMS), dimethyl-3,3'-oxydipropionimide (DODP), dimethyl-3,3'-(methylenedioxy)dipropionimide (DMDP), dimethyl-3,3'-(dimethylenedioxy)dipropionimide (DDDP), dimethyl-3,3'-(tetramethylenedioxy)-  
 25 dipropionimide (DTDP), and dimethyl-3,3'-dithiobispropionimide (DTBP).

Preferred, non-limiting examples of homobifunctional isothiocyanates include: p-phenylenediisothiocyanate (DITC), and 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS).

Preferred, non-limiting examples of homobifunctional isocyanates include xylene-  
 30 diisocyanate, toluene-2,4-diisocyanate, toluene-2-isocyanate-4-isothiocyanate, 3-

methoxydiphenylmethane-4,4'-diisocyanate, 2,2'-dicarboxy-4,4'-azophenyldiisocyanate, and hexamethylenediisocyanate.

Preferred, non-limiting examples of homobifunctional arylhalides include 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and 4,4'-difluoro-3,3'-dinitrophenyl-sulfone.

5 Preferred, non-limiting examples of homobifunctional aliphatic aldehyde reagents include glyoxal, malondialdehyde, and glutaraldehyde.

Preferred, non-limiting examples of homobifunctional acylating reagents include nitrophenyl esters of dicarboxylic acids.

Preferred, non-limiting examples of homobifunctional aromatic sulfonyl chlorides  
10 include phenol-2,4-disulfonyl chloride, and  $\alpha$ -naphthol-2,4-disulfonyl chloride.

Preferred, non-limiting examples of additional amino-reactive homobifunctional reagents include erythritolbiscarbonate which reacts with amines to give biscarbamates.

#### 15 b. Homobifunctional Crosslinkers Reactive with Free Sulfhydryl Groups

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

20 Preferred, non-limiting examples of homobifunctional maleimides include bismaleimido-hexane (BMH), N,N'-(1,3-phenylene) bismaleimide, N,N'-(1,2-phenylene)bismaleimide, azophenyldimaleimide, and bis(N-maleimidomethyl)ether.

Preferred, non-limiting examples of homobifunctional pyridyl disulfides include 1,4-di-3'-(2'-pyridyldithio)propionamidobutane (DPDPB).

25 Preferred, non-limiting examples of homobifunctional alkyl halides include 2,2'-dicarboxy-4,4'-diiodoacetamidoazobenzene,  $\alpha,\alpha'$ -diiodo-p-xylenesulfonic acid,  $\alpha,\alpha'$ -dibromo-p-xylenesulfonic acid, N,N'-bis(b-bromoethyl)benzylamine, N,N'-di(bromoacetyl)phenylhydrazine, and 1,2-di(bromoacetyl)amino-3-phenylpropane.

### c. Homobifunctional Photoactivatable Crosslinkers

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Some of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of homobifunctional photoactivatable crosslinker include bis- $\beta$ -(4-azidosalicylamido)ethyldisulfide (BASED), di-N-(2-nitro-4-azidophenyl)-cystamine-S,S-dioxide (DNCO), and 4,4'-dithiobisphenylazide.

## 5. HeteroBifunctional Reagents

### a. Amino-Reactive HeteroBifunctional Reagents with a Pyridyl Disulfide Moiety

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of hetero-bifunctional reagents with a pyridyl disulfide moiety and an amino-reactive NHS ester include N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), succinimidyl 6-3-(2-pyridyldithio)propionamidohexanoate (LC-SPDP), sulfosuccinimidyl 6-3-(2-pyridyldithio)propionamidohexanoate (sulfo-LCSPDP), 4-succinimidylloxycarbonyl- $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluene (SMPT), and sulfosuccinimidyl 6- $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluamidohexanoate (sulfo-LC-SMPT).

### b. Amino-Reactive HeteroBifunctional Reagents with a Maleimide Moiety

Synthesis, properties, and applications of such reagents are described in the literature. Preferred, non-limiting examples of hetero-bifunctional reagents with a maleimide moiety and an amino-reactive NHS ester include succinimidyl maleimidylacetate (AMAS), succinimidyl 3-maleimidylpropionate (BMPS), N- $\gamma$ -maleimidobutyryloxysuccinimide ester (GMBS) N- $\gamma$ -maleimidobutyryloxysulfo succinimide ester (sulfo-GMBS) succinimidyl 6-maleimidylhexanoate (EMCS), succinimidyl 3-maleimidylbenzoate (SMB),

m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS), succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), and  
 5 sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (sulfo-SMPB).

c. Amino-Reactive HeteroBifunctional Reagents with an Alkyl Halide Moiety

Synthesis, properties, and applications of such reagents are described in the literature.  
 10 Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive NHS ester include N-succinimidyl-(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl-(4-iodoacetyl)aminobenzoate (sulfo-SIAB), succinimidyl-6-(iodoacetyl)aminohexanoate (SIAX), succinimidyl-6-(6-((iodoacetyl)-amino)hexanoylamino)hexanoate (SIAXX), succinimidyl-6-(((4-(iodoacetyl)-amino)-  
 15 methyl)-cyclohexane-1-carbonyl)aminohexanoate (SIACX), and succinimidyl-4((iodoacetyl)-amino)methylcyclohexane-1-carboxylate (SIAC).

A preferred example of a hetero-bifunctional reagent with an amino-reactive NHS ester and an alkyl dihalide moiety is N-hydroxysuccinimidyl 2,3-dibromopropionate (SDBP). SDBP introduces intramolecular crosslinks to the affinity component by conjugating its  
 20 amino groups. The reactivity of the dibromopropionyl moiety towards primary amine groups is controlled by the reaction temperature (McKenzie *et al.*, *Protein Chem.* 7: 581-592 (1988)).

Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive p-nitrophenyl ester moiety include p-nitrophenyl iodoacetate  
 25 (NPPIA).

Other cross-linking agents are known to those of skill in the art. See, for example, Pomato *et al.*, U.S. Patent No. 5,965,106. It is within the abilities of one of skill in the art to choose an appropriate cross-linking agent for a particular application.

#### d. Cleavable Linker Groups

In yet a further embodiment, the linker group is provided with a group that can be cleaved to release the modifying group from the sugar residue. Many cleavable groups are known in the art. See, for example, Jung *et al.*, *Biochem. Biophys. Acta* 761: 152-162 (1983);  
5 Joshi *et al.*, *J. Biol. Chem.* 265: 14518-14525 (1990); Zarling *et al.*, *J. Immunol.* 124: 913-920 (1980); Bouizar *et al.*, *Eur. J. Biochem.* 155: 141-147 (1986); Park *et al.*, *J. Biol. Chem.* 261: 205-210 (1986); Browning *et al.*, *J. Immunol.* 143: 1859-1867 (1989). Moreover a broad range of cleavable, bifunctional (both homo- and hetero-bifunctional) linker groups is commercially available from suppliers such as Pierce.

10 Exemplary cleavable moieties can be cleaved using light, heat or reagents such as thiols, hydroxylamine, bases, periodate and the like. Moreover, certain preferred groups are cleaved *in vivo* in response to being endocytosed (e.g., cis-aconityl; see, Shen *et al.*, *Biochem. Biophys. Res. Commun.* 102: 1048 (1991)). Preferred cleavable groups comprise a cleavable moiety which is a member selected from the group consisting of disulfide, ester, imide,  
15 carbonate, nitrobenzyl, phenacyl and benzoin groups.

#### e. Conjugation of Modified Sugars to Peptides

The modified sugars are conjugated to a glycosylated or non-glycosylated peptide using an appropriate enzyme to mediate the conjugation. Preferably, the concentrations of  
20 the modified donor sugar(s), enzyme(s) and acceptor peptide(s) are selected such that glycosylation proceeds until the acceptor is consumed. The considerations discussed below, while set forth in the context of a sialyltransferase, are generally applicable to other glycosyltransferase reactions.

A number of methods of using glycosyltransferases to synthesize desired  
25 oligosaccharide structures are known and are generally applicable to the instant invention. Exemplary methods are described, for instance, WO 96/32491, Ito *et al.*, *Pure Appl. Chem.* 65: 753 (1993), and U.S. Pat. Nos. 5,352,670, 5,374,541, and 5,545,553.

The present invention is practiced using a single glycosyltransferase or a combination of glycosyltransferases. For example, one can use a combination of a sialyltransferase and a  
30 galactosyltransferase. In those embodiments using more than one enzyme, the enzymes and substrates are preferably combined in an initial reaction mixture, or the enzymes and reagents

for a second enzymatic reaction are added to the reaction medium once the first enzymatic reaction is complete or nearly complete. By conducting two enzymatic reactions in sequence in a single vessel, overall yields are improved over procedures in which an intermediate species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is reduced.

In a preferred embodiment, each of the first and second enzyme is a glycosyltransferase. In another preferred embodiment, one enzyme is an endoglycosidase. In another preferred embodiment, one enzyme is an exoglycosidase. In an additional preferred embodiment, more than two enzymes are used to assemble the modified glycoprotein of the invention. The enzymes are used to alter a saccharide structure on the peptide at any point either before or after the addition of the modified sugar to the peptide.

In another embodiment, at least two of the enzymes are glycosyltransferases and the last sugar added to the saccharide structure of the peptide is a non-modified sugar. Instead, the modified sugar is internal to the glycan structure and therefore need not be the ultimate sugar on the glycan. In an exemplary embodiment, galactosyltransferase may catalyze the transfer of Gal-PEG from UDP-Gal-PEG onto the glycan, followed by incubation in the presence of ST3Gal3 and CMP-SA, which serves to add a "capping" unmodified sialic acid onto the glycan (Figure 23A).

In another embodiment, at least two of the enzymes used are glycosyltransferases, and at least two modified sugars are added to the glycan structures on the peptide. In this manner, two or more different glycoconjugates may be added to one or more glycans on a peptide. This process generates glycan structures having two or more functionally different modified sugars. In an exemplary embodiment, incubation of the peptide with GnT-I, II and UDP-GlcNAc-PEG serves to add a GlcNAc-PEG molecule to the glycan; incubation with galactosyltransferase and UDP-Gal then serves to add a Gal residue thereto; and, incubation with ST3Gal3 and CMP-SA-Man-6-Phosphate serves to add a SA-mannose-6-phosphate molecule to the glycan. This series of reactions results in a glycan chain having the functional characteristics of a PEGylated glycan as well as mannose-6-phosphate targeting activity (Figure 23B).

In another embodiment, at least two of the enzymes used in the reaction are glycosyltransferases, and again, different modified sugars are added to N-linked and O-



linked glycans on the peptide. This embodiment is useful when two different modified sugars are to be added to the glycans of a peptide, but when it is important to spatially separate the modified sugars on the peptide from each other. For example, if the modified sugars comprise bulky molecules, including but not limited to, PEG and other molecules such as a linker molecule, this method may be preferable. The modified sugars may be added simultaneously to the glycan structures on a peptide, or they may be added sequentially. In an exemplary embodiment, incubation with ST3Gal3 and CMP-SA-PEG serves to add sialic acid-PEG to the N-linked glycans, while incubation with ST3Gal1 and CMP-SA-bisPhosphonate serves to add sialic acid-BisPhosphonate to the O-linked glycans (Figure 23C).

In another embodiment, the method makes use of one or more exo- or endoglycosidase. The glycosidase is typically a mutant, which is engineered to form glycosyl bonds rather than rupture them. The mutant glycanase, sometimes called a glycosynthase, typically includes a substitution of an amino acid residue for an active site acidic amino acid residue. For example, when the endoglycanase is endo-H, the substituted active site residues will typically be Asp at position 130, Glu at position 132 or a combination thereof. The amino acids are generally replaced with serine, alanine, asparagine, or glutamine. Exoglycosidases such as transialylidase are also useful.

The mutant enzyme catalyzes the reaction, usually by a synthesis step that is analogous to the reverse reaction of the endoglycanase hydrolysis step. In these embodiments, the glycosyl donor molecule (*e.g.*, a desired oligo- or mono-saccharide structure) contains a leaving group and the reaction proceeds with the addition of the donor molecule to a GlcNAc residue on the protein. For example, the leaving group can be a halogen, such as fluoride. In other embodiments, the leaving group is a Asn, or a Asn-peptide moiety. In yet further embodiments, the GlcNAc residue on the glycosyl donor molecule is modified. For example, the GlcNAc residue may comprise a 1,2 oxazoline moiety.

In a preferred embodiment, each of the enzymes utilized to produce a conjugate of the invention are present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to reaction conditions such as temperature, time and pH value. Means for determining the catalytic

amount for a given enzyme under preselected substrate concentrations and reaction conditions are well known to those of skill in the art.

The temperature at which an above-described process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures.

5 Preferred temperature ranges are about 0 °C to about 55 °C, and more preferably about 20 °C to about 37 °C. In another exemplary embodiment, one or more components of the present method are conducted at an elevated temperature using a thermophilic enzyme.

The reaction mixture is maintained for a period of time sufficient for the acceptor to be glycosylated, thereby forming the desired conjugate. Some of the conjugate can often be  
10 detected after a few hours, with recoverable amounts usually being obtained within 24 hours or less. Those of skill in the art understand that the rate of reaction is dependent on a number of variable factors (*e.g.*, enzyme concentration, donor concentration, acceptor concentration, temperature, solvent volume), which are optimized for a selected system.

The present invention also provides for the industrial-scale production of modified  
15 peptides. As used herein, an industrial scale generally produces at least one gram of finished, purified conjugate.

In the discussion that follows, the invention is exemplified by the conjugation of modified sialic acid moieties to a glycosylated peptide. The exemplary modified sialic acid is labeled with PEG. The focus of the following discussion on the use of PEG-modified sialic  
20 acid and glycosylated peptides is for clarity of illustration and is not intended to imply that the invention is limited to the conjugation of these two partners. One of skill understands that the discussion is generally applicable to the additions of modified glycosyl moieties other than sialic acid. Moreover, the discussion is equally applicable to the modification of a glycosyl unit with agents other than PEG including other water-soluble polymers, therapeutic  
25 moieties, and biomolecules.

An enzymatic approach can be used for the selective introduction of PEGylated or PPGylated carbohydrates onto a peptide or glycopeptide. The method utilizes modified sugars containing PEG, PPG, or a masked reactive functional group, and is combined with the appropriate glycosyltransferase or glycosynthase. By selecting the glycosyltransferase  
30 that will make the desired carbohydrate linkage and utilizing the modified sugar as the donor substrate, the PEG or PPG can be introduced directly onto the peptide backbone, onto

existing sugar residues of a glycopeptide or onto sugar residues that have been added to a peptide.

An acceptor for the sialyltransferase is present on the peptide to be modified by the methods of the present invention either as a naturally occurring structure or one placed there recombiantly, enzymatically or chemically. Suitable acceptors, include, for example, galactosyl acceptors such as Gal $\beta$ 1,4GlcNAc, Gal $\beta$ 1,4GalNAc, Gal $\beta$ 1,3GalNAc, lacto-N-tetraose, Gal $\beta$ 1,3GlcNAc, Gal $\beta$ 1,3Ara, Gal $\beta$ 1,6GlcNAc, Gal $\beta$ 1,4Glc (lactose), and other acceptors known to those of skill in the art (*see, e.g., Paulson et al., J. Biol. Chem.* 253: 5617-5624 (1978)).

In one embodiment, an acceptor for the sialyltransferase is present on the peptide to be modified upon *in vivo* synthesis of the peptide. Such peptides can be sialylated using the claimed methods without prior modification of the glycosylation pattern of the peptide. Alternatively, the methods of the invention can be used to sialylate a peptide that does not include a suitable acceptor; one first modifies the peptide to include an acceptor by methods known to those of skill in the art. In an exemplary embodiment, a GalNAc residue is added by the action of a GalNAc transferase.

In an exemplary embodiment, the galactosyl acceptor is assembled by attaching a galactose residue to an appropriate acceptor linked to the peptide, e.g., a GlcNAc. The method includes incubating the peptide to be modified with a reaction mixture that contains a suitable amount of a galactosyltransferase (*e.g., gal $\beta$ 1,3 or gal $\beta$ 1,4*), and a suitable galactosyl donor (*e.g., UDP-galactose*). The reaction is allowed to proceed substantially to completion or, alternatively, the reaction is terminated when a preselected amount of the galactose residue is added. Other methods of assembling a selected saccharide acceptor will be apparent to those of skill in the art.

In yet another embodiment, peptide-linked oligosaccharides are first "trimmed," either in whole or in part, to expose either an acceptor for the sialyltransferase or a moiety to which one or more appropriate residues can be added to obtain a suitable acceptor. Enzymes such as glycosyltransferases and endoglycosidases (*see, for example U.S. Patent No. 5,716,812*) are useful for the attaching and trimming reactions. A detailed discussion of "trimming" and remodeling N-linked and O-linked glycans is provided elsewhere herein.

In the discussion that follows, the method of the invention is exemplified by the use of modified sugars having a water-soluble polymer attached thereto. The focus of the discussion is for clarity of illustration. Those of skill will appreciate that the discussion is equally relevant to those embodiments in which the modified sugar bears a therapeutic moiety, biomolecule or the like.

An exemplary embodiment of the invention in which a carbohydrate residue is "trimmed" prior to the addition of the modified sugar is set forth in Figure 14, which sets forth a scheme in which high mannose is trimmed back to the first generation biantennary structure. A modified sugar bearing a water-soluble polymer is conjugated to one or more of the sugar residues exposed by the "trimming back." In one example, a water-soluble polymer is added via a GlcNAc moiety conjugated to the water-soluble polymer. The modified GlcNAc is attached to one or both of the terminal mannose residues of the biantennary structure. Alternatively, an unmodified GlcNAc can be added to one or both of the termini of the branched species.

In another exemplary embodiment, a water-soluble polymer is added to one or both of the terminal mannose residues of the biantennary structure via a modified sugar having a galactose residue, which is conjugated to a GlcNAc residue added onto the terminal mannose residues. Alternatively, an unmodified Gal can be added to one or both terminal GlcNAc residues.

In yet a further example, a water-soluble polymer is added onto a Gal residue using a modified sialic acid.

Another exemplary embodiment is set forth in Figure 15, which displays a scheme similar to that shown in Figure 14, in which the high mannose structure is "trimmed back" to the mannose from which the biantennary structure branches. In one example, a water-soluble polymer is added via a GlcNAc modified with the polymer. Alternatively, an unmodified GlcNAc is added to the mannose, followed by a Gal with an attached water-soluble polymer. In yet another embodiment, unmodified GlcNAc and Gal residues are sequentially added to the mannose, followed by a sialic acid moiety modified with a water-soluble polymer.

Figure 16 sets forth a further exemplary embodiment using a scheme similar to that shown in Figure 14, in which high mannose is "trimmed back" to the GlcNAc to which the first mannose is attached. The GlcNAc is conjugated to a Gal residue bearing a water-soluble

polymer. Alternatively, an unmodified Gal is added to the GlcNAc, followed by the addition of a sialic acid modified with a water-soluble sugar. In yet a further example, the terminal GlcNAc is conjugated with Gal and the GlcNAc is subsequently fucosylated with a modified fucose bearing a water-soluble polymer.

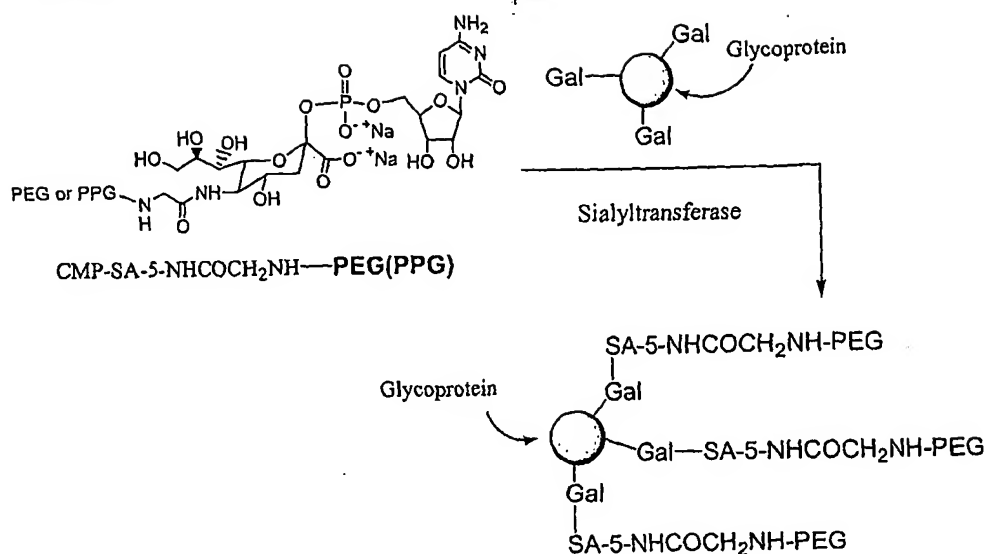
5        Figure 17 is a scheme similar to that shown in Figure 14, in which high mannose is trimmed back to the first GlcNAc attached to the Asn of the peptide. In one example, the GlcNAc of the GlcNAc-(Fuc)<sub>a</sub> residue is conjugated with a GlcNAc bearing a water soluble polymer. In another example, the GlcNAc of the GlcNAc-(Fuc)<sub>a</sub> residue is modified with Gal, which bears a water soluble polymer. In a still further embodiment, the GlcNAc is  
10        modified with Gal, followed by conjugation to the Gal of a sialic acid modified with a water-soluble polymer.

Other exemplary embodiments are set forth in Figures 18-22. An illustration of the array of reaction types with which the present invention may be practiced is provided in each of the aforementioned figures.

15        The Examples set forth above provide an illustration of the power of the methods set forth herein. Using the methods of the invention, it is possible to "trim back" and build up a carbohydrate residue of substantially any desired structure. The modified sugar can be added to the termini of the carbohydrate moiety as set forth above, or it can be intermediate between the peptide core and the terminus of the carbohydrate.

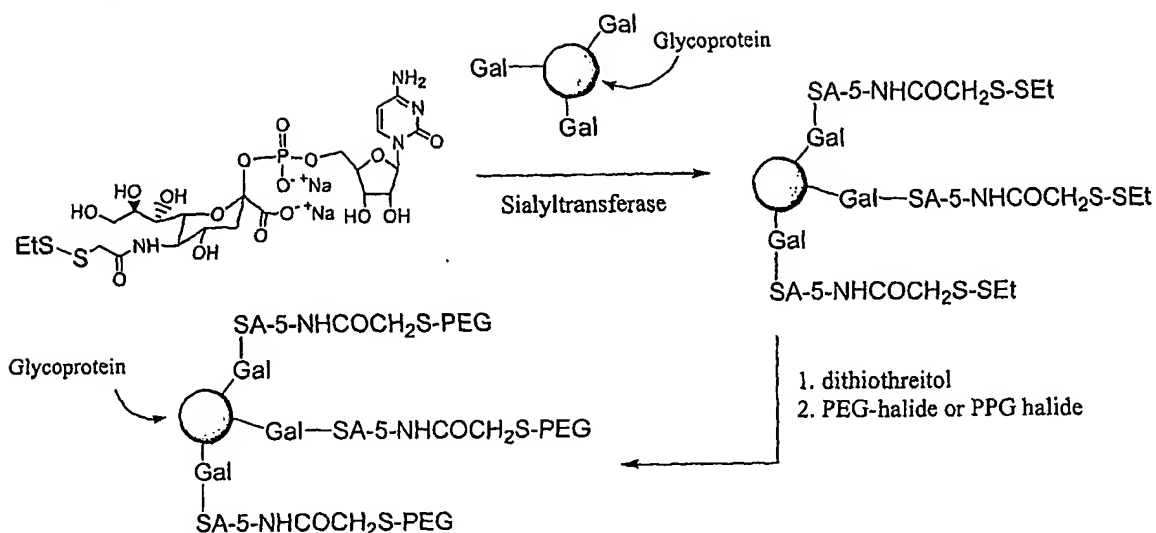
20        In an exemplary embodiment, an existing sialic acid is removed from a glycopeptide using a sialidase, thereby unmasking all or most of the underlying galactosyl residues. Alternatively, a peptide or glycopeptide is labeled with galactose residues, or an oligosaccharide residue that terminates in a galactose unit. Following the exposure of or  
25        addition of the galactose residues, an appropriate sialyltransferase is used to add a modified sialic acid. The approach is summarized in Scheme 12.

Scheme 12



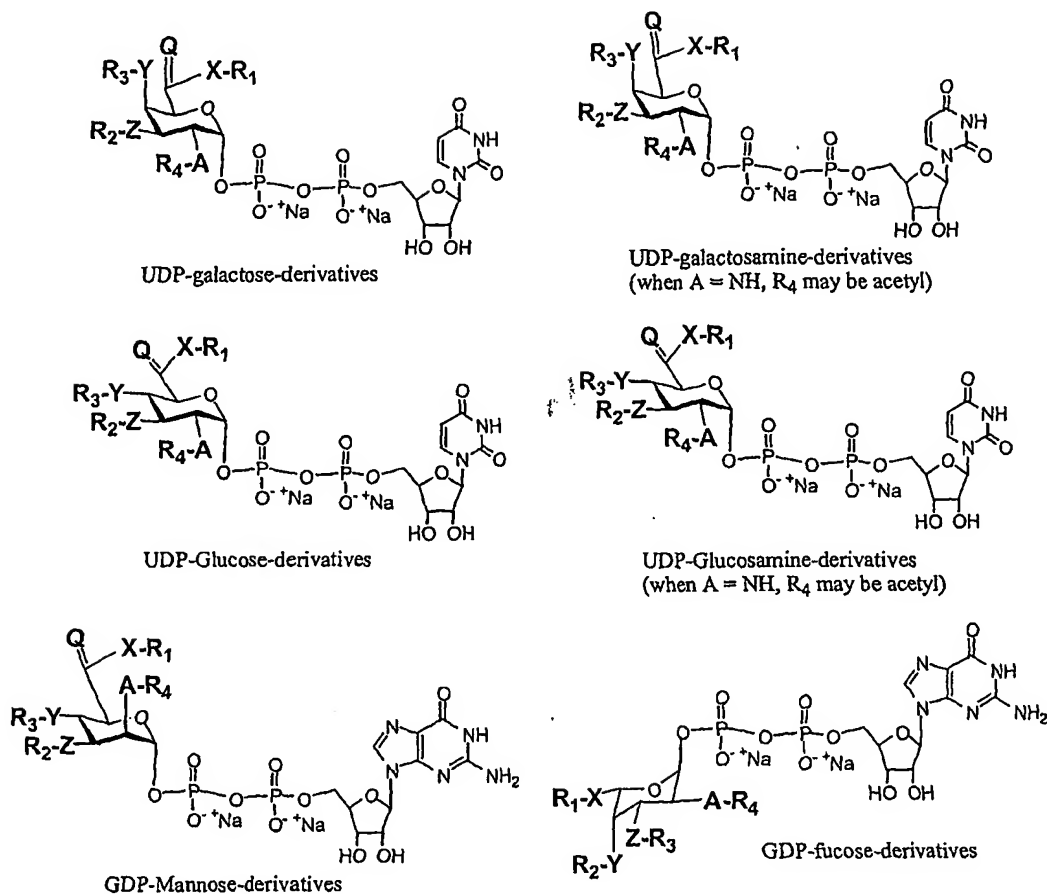
In yet a further approach, summarized in Scheme 13, a masked reactive functionality is present on the sialic acid. The masked reactive group is preferably unaffected by the conditions used to attach the modified sialic acid to the peptide. After the covalent attachment of the modified sialic acid to the peptide, the mask is removed and the peptide is conjugated with an agent such as PEG, PPG, a therapeutic moiety, biomolecule or other agent. The agent is conjugated to the peptide in a specific manner by its reaction with the unmasked reactive group on the modified sugar residue.

Scheme 13



- 5 Any modified sugar can be used with its appropriate glycosyltransferase, depending on the terminal sugars of the oligosaccharide side chains of the glycopeptide (Table 4). As discussed above, the terminal sugar of the glycopeptide required for introduction of the PEGylated or PPGylated structure can be introduced naturally during expression or it can be produced post expression using the appropriate glycosidase(s), glycosyltransferase(s) or mix
- 10 of glycosidase(s) and glycosyltransferase(s).

Table 4. Modified sugars.

X = O, NH, S, CH<sub>2</sub>, N-(R<sub>1-5</sub>)<sub>2</sub>.

Y = X; Z = X; A = X; B = X.

Q = H<sub>2</sub>, O, S, NH, N-R.R, R<sub>1-4</sub> = H, Linker-M, M.

M = Ligand of interest

Ligand of interest = acyl-PEG, acyl-PPG, alkyl-PEG, acyl-alkyl-PEG, acyl-alkyl-PEG, carbamoyl-PEG, carbamoyl-PPG, PEG, PPG, acyl-aryl-PEG, acyl-aryl-PPG, aryl-PEG, aryl-PPG, Mannose- $\gamma$ -phosphate, heparin, heparan, SLex, Mannose, FGF, VFGF, protein, chondroitin, keratan, dermatan, albumin, integrins, peptides, etc.

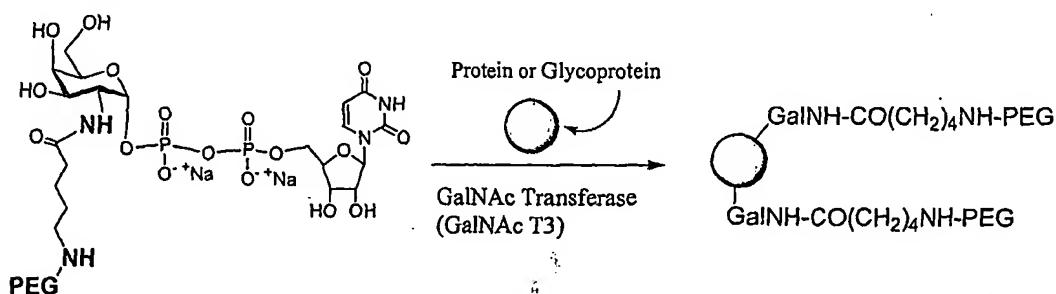
- In a further exemplary embodiment, UDP-galactose-PEG is reacted with bovine milk
- 5  $\beta$ 1,4-galactosyltransferase, thereby transferring the modified galactose to the appropriate terminal N-acetylglucosamine structure. The terminal GlcNAc residues on the glycopeptide may be produced during expression, as may occur in such expression systems as mammalian, insect, plant or fungus, but also can be produced by treating the glycopeptide with a sialidase and/or glycosidase and/or glycosyltransferase, as required.



In another exemplary embodiment, a GlcNAc transferase, such as GnT-I-IV, is utilized to transfer PEGylated-GlcNAc to a mannose residue on a glycopeptide. In a still further exemplary embodiment, the N- and/or O-linked glycan structures are enzymatically removed from a glycopeptide to expose an amino acid or a terminal glycosyl residue that is subsequently conjugated with the modified sugar. For example, an endoglycanase is used to remove the N-linked structures of a glycopeptide to expose a terminal GlcNAc as a GlcNAc-linked-Asn on the glycopeptide. UDP-Gal-PEG and the appropriate galactosyltransferase is used to introduce the PEG- or PPG-galactose functionality onto the exposed GlcNAc.

In an alternative embodiment, the modified sugar is added directly to the peptide backbone using a glycosyltransferase known to transfer sugar residues to the peptide backbone. This exemplary embodiment is set forth in Scheme 14. Exemplary glycosyltransferases useful in practicing the present invention include, but are not limited to, GalNAc transferases (GalNAc T1-14), GlcNAc transferases, fucosyltransferases, glucosyltransferases, xylosyltransferases, mannosyltransferases and the like. Use of this approach allows the direct addition of modified sugars onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the modified sugar occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the peptide chain.

Scheme 14



In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the

modified sugar to the peptide. In an exemplary embodiment, an enzyme (*e.g.*, fucosyltransferase) is used to append a glycosyl unit (*e.g.*, fucose) onto the terminal modified sugar attached to the peptide. In another example, an enzymatic reaction is utilized to “cap” sites to which the modified sugar failed to conjugate. Alternatively, a chemical reaction is utilized to alter the structure of the conjugated modified sugar. For example, the conjugated modified sugar is reacted with agents that stabilize or destabilize its linkage with the peptide component to which the modified sugar is attached. In another example, a component of the modified sugar is deprotected following its conjugation to the peptide. One of skill will appreciate that there is an array of enzymatic and chemical procedures that are useful in the methods of the invention at a stage after the modified sugar is conjugated to the peptide. Further elaboration of the modified sugar-peptide conjugate is within the scope of the invention.

#### Peptide Targeting With Mannose-6-Phosphate

In an exemplary embodiment the peptide is derivatized with at least one mannose-6-phosphate moiety. The mannose-6-phosphate moiety targets the peptide to a lysosome of a cell, and is useful, for example, to target therapeutic proteins to lysosomes for therapy of lysosomal storage diseases.

Lysosomal storage diseases are a group of over 40 disorders which are the result of defects in genes encoding enzymes that break down glycolipid or polysaccharide waste products within the lysosomes of cells. The enzymatic products, *e.g.*, sugars and lipids, are then recycled into new products. Each of these disorders results from an inherited autosomal or X-linked recessive trait which affects the levels of enzymes in the lysosome. Generally, there is no biological or functional activity of the affected enzymes in the cells and tissues of affected individuals. Table 5 provides a list of representative storage diseases and the enzymatic defect associated with the diseases. In such diseases the deficiency in enzyme function creates a progressive systemic deposition of lipid or carbohydrate substrate in lysosomes in cells in the body, eventually causing loss of organ function and death. The genetic etiology, clinical manifestations, molecular biology and possibility of the lysosomal storage diseases are detailed in Scriver *et al.*, eds., *THE METABOLIC AND MOLECULAR BASIS OF INHERITED DISEASE*, 7<sup>sup</sup>.th Ed., Vol. II, McGraw Hill, (1995).

**Table 5.** Lysosomal storage diseases and associated enzymatic defects

Disease	Enzymatic Defect
Pompe disease	acid $\alpha$ -glucosidase (acid maltase)
MPSI* (Hurler disease)	$\alpha$ -L-iduronidase
MPSII (Hunter disease)	iduronate sulfatase
MPSIII (Sanfilippo)	heparan N-sulfatase
MPS IV (Morquio A)	galactose-6-sulfatase
MPS IV (Morquio B)	acid $\beta$ -galactosidase
MPS VII (Sly disease)	$\beta$ -glucuronidase
I-cell disease	N-acetylglucosamine-1-phosphotransferase
Schindler disease	$\alpha$ -N-acetylgalactosaminidase ( $\alpha$ -galactosidase B)
Wolman disease	acid lipase
Cholesterol ester storage disease	acid lipase
Farber disease	lysosomal acid ceramidase
Niemann-Pick disease	acid sphingomyelinase
Gaucher disease	glucocerebrosidase
Krabbe disease	galactosylceramidase
Fabry disease	$\alpha$ -galactosidase A
GM1 gangliosidosis	acid $\beta$ -galactosidase
Galactosialidosis	$\beta$ -galactosidase and neuraminidase
Tay-Sach's disease	hexosaminidase A
Magakaryotic leukodystrophy	arylsulphatase a
Sandhoff disease	hexosaminidase A and B

\*MPS = mucopolysaccharidosis

De Duve first suggested that replacement of the missing lysosomal enzyme with exogenous biologically active enzyme might be a viable approach to treatment of lysosomal storage diseases (De Duve, *Fed. Proc.* 23: 1045 (1964). Since that time, various studies have suggested that enzyme replacement therapy may be beneficial for treating various lysosomal storage diseases. The best success has been shown with individuals with type I Gaucher disease, who have been treated with exogenous enzyme ( $\beta$ -glucocerebrosidase), prepared from placenta (Ceredase™) or, more recently, recombinantly (Cerezyme™). It has been suggested that enzyme replacement may also be beneficial for treating Fabry's disease, as well as other lysosomal storage diseases. See, for example, Dawson *et al.*, *Ped. Res.* 7(8): 684-690 (1973) (*in vitro*) and Mapes *et al.*, *Science* 169: 987 (1970) (*in vivo*). Clinical trials of enzyme replacement therapy have been reported for Fabry patients using infusions of normal plasma (Mapes *et al.*, *Science* 169: 987-989 (1970)),  $\alpha$ -galactosidase A purified from placenta (Brady *et al.*, *N. Eng. J. Med.* 279: 1163 (1973)); or  $\alpha$ -galactosidase A purified from spleen or plasma (Desnick *et al.*, *Proc. Natl. Acad. Sci., USA* 76: 5326-5330 (1979)) and have demonstrated the biochemical effectiveness of direct enzyme replacement for Fabry disease.

These studies indicate the potential for eliminating, or significantly reducing, the pathological glycolipid storage by repeated enzyme replacement. For example, in one study (Desnick *et al.*, *supra*), intravenous injection of purified enzyme resulted in a transient reduction in the plasma levels of the stored lipid substrate, globotriasylceramide.

5 Accordingly, there exists a need in the art for methods for providing sufficient quantities of biologically active lysosomal enzymes, such as human  $\alpha$ -galactosidase A, to deficient cells. Recently, recombinant approaches have attempted to address these needs, *see*, e.g., U.S. Pat. No. 5,658,567; 5,580,757; Bishop *et al.*, *Proc. Natl. Acad. Sci., USA*. 83: 4859-4863 (1986); Medin *et al.*, *Proc. Natl. Acad. Sci., USA*. 93: 7917-7922 (1996); Novo, F. J.,  
10 *Gene Therapy*. 4: 488-492 (1997); Ohshima *et al.*, *Proc. Natl. Acad. Sci., USA*. 94: 2540-2544 (1997); and Sugimoto *et al.*, *Human Gene Therapy* 6: 905-915, (1995). Through the mannose-6-phosphate mediated targeting of therapeutic peptides to lysosomes, the present invention provides compositions and methods for delivering sufficient quantities of biologically active lysosomal peptides to deficient cells.

15 Thus, in an exemplary embodiment, the present invention provides a peptide according to Table 7 that is derivatized with mannose-6-phosphate (Figure 24 and Figure 25). The peptide may be recombinantly or chemically prepared. Moreover, the peptide can be the full, natural sequence, or it may be modified by, for example, truncation, extension, or it may include substitutions or deletions. Exemplary proteins that are remodeled using a method of  
20 the present invention include glucocerebrosidase,  $\beta$ -glucosidase,  $\alpha$ -galactosidase A, acid- $\alpha$ -glucosidase (acid maltase). Representative modified peptides that are in clinical use include, but are not limited to, Ceredase<sup>TM</sup>, Cerezyme<sup>TM</sup>, and Fabryzyme<sup>TM</sup>. A glycosyl group on modified and clinically relevant peptides may also be altered utilizing a method of the invention. The mannose-6-phosphate is attached to the peptide via a glycosyl linking group.

25 In an exemplary embodiment, the glycosyl linking group is derived from sialic acid. Exemplary sialic acid-derived glycosyl linking groups are set forth in Table 3, in which one or more of the "R" moieties is mannose-6-phosphate or a spacer group having one or more mannose-6-phosphate moieties attached thereto. The modified sialic acid moiety is preferably the terminal residue of an oligosaccharide linked to the surface of the peptide

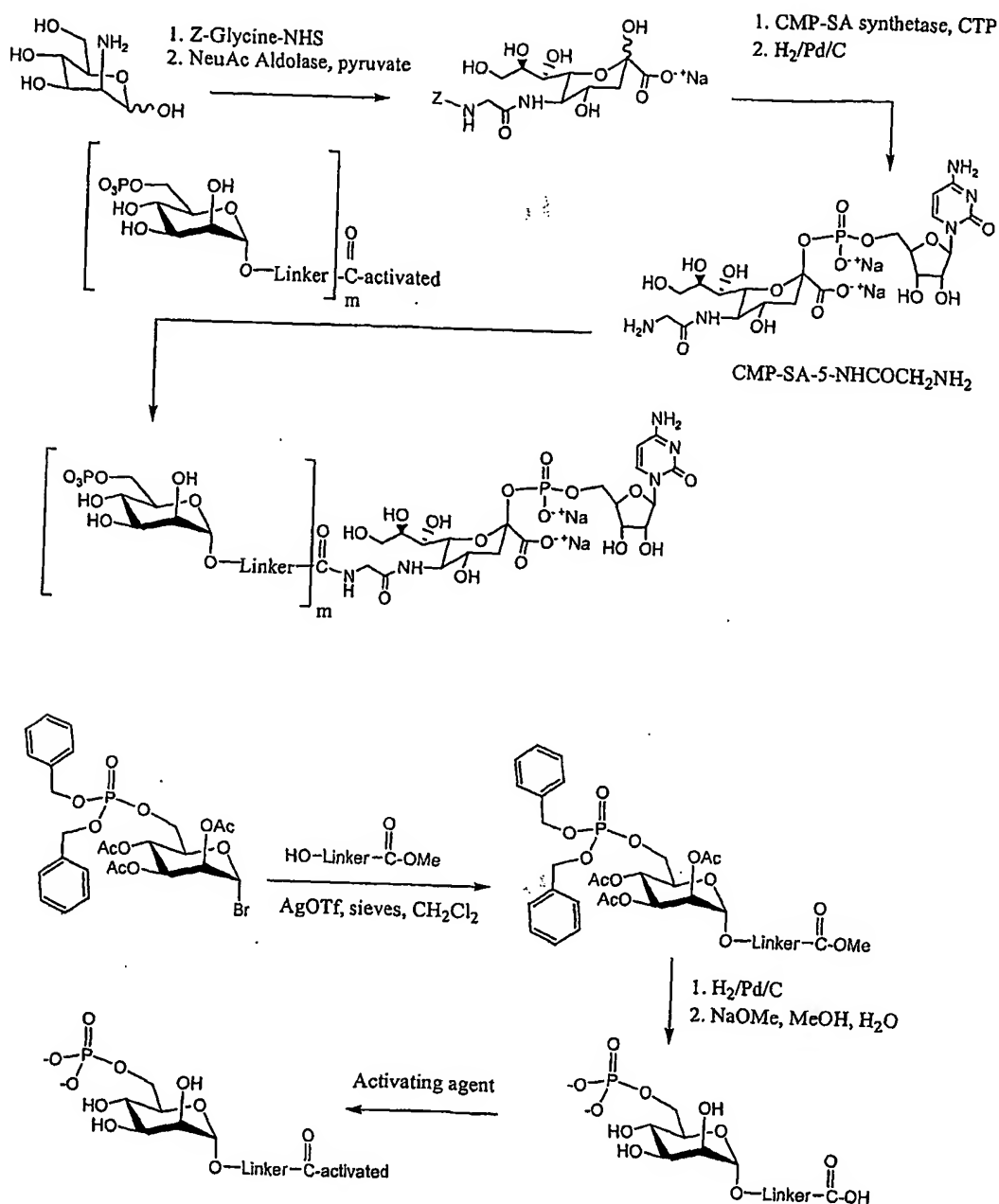
30 (Figure 26)

In addition to the mannose-6-phosphate, the peptides of the invention may be further derivatized with a moiety such as a water-soluble polymer, a therapeutic moiety, or an additional targeting moiety. Methods for attaching these and other groups are set forth herein. In an exemplary embodiment, the group other than mannose-6-phosphate is attached  
5 to the peptide via a derivatized sialic acid derivative according to Table 3, in which one or more of the "R" moieties is a group other than mannose-6-phosphate.

In an exemplary embodiment, a sialic acid moiety modified with a Cbz-protected glycine-based linker arm is prepared. The corresponding nucleotide sugar is prepared and the Cbz group is removed by catalytic hydrogenation. The resulting nucleotide sugar has an  
10 available, reactive amine that is contacted with an activated mannose-6-phosphate derivative, providing a mannose-6-phosphate derivatized nucleotide sugar that is useful in practicing the methods of the invention.

As shown in the scheme below (scheme 15), an exemplary activated mannose-6-phosphate derivative is formed by converting a 2-bromo-benzyl-protected phosphotriester  
15 into the corresponding triflate, *in situ*, and reacting the triflate with a linker having a reactive oxygen-containing moiety, forming an ether linkage between the sugar and the linker. The benzyl protecting groups are removed by catalytic hydrogenation, and the methyl ester of the linker is hydrolyzed, providing the corresponding carboxylic acid. The carboxylic acid is activated by any method known in the art. An exemplary activation procedure relies upon the  
20 conversion of the carboxylic acid to the N-hydroxysuccinimide ester.

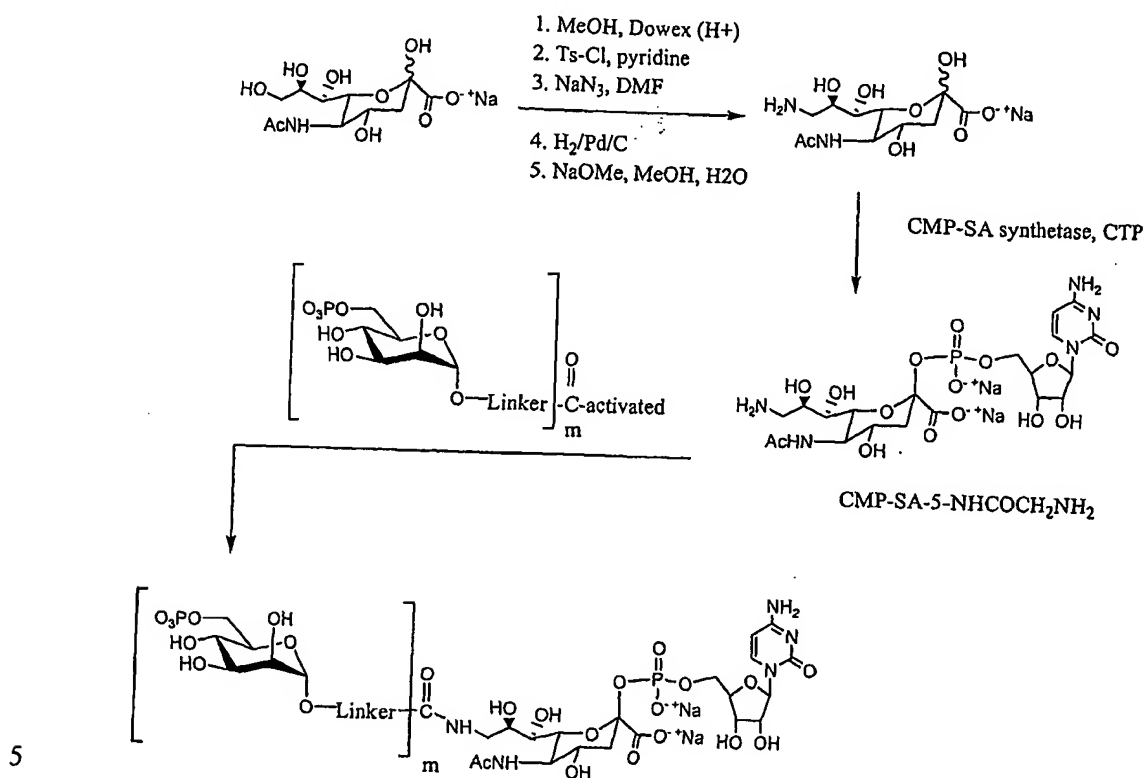
Scheme 15



In another exemplary embodiment, as shown in the scheme below (scheme 16), a N-acetylated sialic acid is converted to an amine by manipulation of the pyruvyl moiety. Thus, the primary hydroxyl is converted to a sulfonate ester and reacted with sodium azide. The azide is catalytically reduced to the corresponding amine. The sugar is

subsequently converted to its nucleotide analogue and coupled, through the amine group, to the linker arm-derivatized mannose-6-phosphate prepared as discussed above.

Scheme 16



Peptides useful to treat lysosomal storage disease can be derivatized with other targeting moieties including, but not limited to, transferrin (to deliver the peptide across the blood-brain barrier, and to endosomes), carnitine (to deliver the peptide to muscle cells), and phosphonates, e.g. bisphosphonate (to target the peptide to bone and other calciferous tissues). The targeting moiety and therapeutic peptide are conjugated by any method discussed herein or otherwise known in the art.

In an exemplary embodiment, the targeting agent and the therapeutic peptide are coupled via a linker moiety. In this embodiment, at least one of the therapeutic peptide or the targeting agent is coupled to the linker moiety via an intact glycosyl linking group according to a method of the invention. In an exemplary embodiment, the linker moiety includes a poly(ether) such as poly(ethylene glycol). In another exemplary embodiment, the linker

moiety includes at least one bond that is degraded *in vivo*, releasing the therapeutic peptide from the targeting agent, following delivery of the conjugate to the targeted tissue or region of the body.

5 In yet another exemplary embodiment, the *in vivo* distribution of the therapeutic moiety is altered via altering a glycoform on the therapeutic moiety without conjugating the therapeutic peptide to a targeting moiety. For example, the therapeutic peptide can be shunted away from uptake by the reticuloendothelial system by capping a terminal galactose moiety of a glycosyl group with sialic acid (or a derivative thereof) (Figures 24 and 27). Sialylation to cover terminal Gal avoids uptake of the peptide by hepatic asialoglycoprotein (ASGP) receptors, and may extend the half life of the peptide as compared with peptides 10 having only complex glycan chains, in the absence of sialylation.

## II. Peptide/Glycopeptides of the Invention

15 In one embodiment, the present invention provides a composition comprising multiple copies of a single peptide having an elemental trimannosyl core as the primary glycan structure attached thereto. In preferred embodiments, the peptide may be a therapeutic molecule. The natural form of the peptide may comprise complex N-linked glycans or may be a high mannose glycan. The peptide may be a mammalian peptide, and is preferably a human peptide. In some embodiments the peptide is selected from the group consisting of an 20 immunoglobulin, erythropoietin, tissue-type activator peptide, and others (See Figure 28).

Exemplary peptides whose glycans can be remodeled using the methods of the invention are set forth in Figure 28.

**Table 6.** Preferred peptides for glycan remodeling



<u>Hormones and Growth Factors</u>	<u>Receptors and Chimeric Receptors</u>
G-CSF	CD4
GM-CSF	Tumor Necrosis Factor receptor (TNF-R)
TPO	TNF-R:IgG Fc fusion
EPO	Alpha-CD20
EPO variants	PSGL-1
FSH	Complement
HGH	GlyCAM or its chimera
insulin	N-CAM or its chimera
alpha-TNF	<u>Monoclonal Antibodies (Immunoglobulins)</u>
Leptin	MAB-anti-RSV
human chorionic gonadotropin	MAB-anti-IL-2 receptor
<u>Enzymes and Inhibitors</u>	MAB-anti-CEA
TPA	MAB-anti-glycoprotein IIb/IIIa
TPA variants	MAB-anti-EGF
Urokinase	MAB-anti-Her2
Factors VII, VIII, IX, X	MAB-CD20
DNase	MAB-alpha-CD3
Glucocerebrosidase	MAB-TNF $\alpha$
Hirudin	MAB-CD4
$\alpha$ 1 antitrypsin ( $\alpha$ 1 protease inhibitor)	MAB-PSGL-1
Antithrombin III	MAB-anti F protein of Respiratory Syncytial Virus
Acid $\alpha$ -glucosidase (acid maltase)	Anti-thrombin-III
$\alpha$ galactosidase A	<u>Cells</u>
$\alpha$ -L-iduronidase	Red blood cells
Urokinase	White blood cells (e.g., T cells, B cells, dendritic cells, macrophages, NK cells, neutrophils, monocytes and the like)
<u>Cytokines and Chimeric Cytokines</u>	Stem cells
Interleukin-1 (IL-1), 1B, 2, 3, 4	Others
Interferon-alpha (IFN-alpha)	Hepatitis B surface antigen (HbsAg)
IFN-alpha-2b	
IFN-beta	
IFN-gamma	
IFN-omega	
Chimeric diphtheria toxin-IL-2	

Table 7. Most preferred peptides for glycan remodeling

Alpha-galactosidase A	Interleukin-2 (IL-2)
Alpha-L-iduronidase	Factor VIII
Anti-thrombin-III	hrDNase
Granulocyte colony stimulating factor (G-CSF)	Insulin
Interferon $\alpha$	Hepatitis B surface protein (HbsAg)
Interferon $\beta$	Human Growth Hormone (HGH)
Interferon omega	Human chorionic gonadotropin
	Urokinase

Factor VII clotting factor	TNF receptor-IgG Fc fusion (Enbrel™)
Factor IX clotting factor	MAB-Her-2 (Herceptin™)
Follicle Stimulating Hormone (FSH)	MAB-F protein of Respiratory
Erythropoietin (EPO)	Syncytial Virus (Synagis™)
Granulocyte-macrophage colony stimulating factor (GM-CSF)	MAB-CD20 (Rituxan™)
Interferon $\gamma$	MAB-TNF $\alpha$ (Remicade™)
$\alpha_1$ protease inhibitor ( $\alpha_1$ antitrypsin)	MAB-Glycoprotein IIb/IIIa (Reopro™)
Tissue-type plasminogen activator (TPA)	
Glucocerebrosidase (Cerezyme™)	

A more detailed list of peptides useful in the invention and their source is provided in Figure 28.

Other exemplary peptides that are modified by the methods of the invention include members of the immunoglobulin family (*e.g.*, antibodies, MHC molecules, T cell receptors, and the like), intercellular receptors (*e.g.*, integrins, receptors for hormones or growth factors and the like) lectins, and cytokines (*e.g.*, interleukins). Additional examples include tissue-type plasminogen activator (TPA), renin, clotting factors such as Factor VIII and Factor IX, bombesin, thrombin, hematopoietic growth factor, colony stimulating factors, viral antigens, complement peptides,  $\alpha_1$ -antitrypsin, erythropoietin, P-selectin glycopeptide ligand-1 (PSGL-1), granulocyte-macrophage colony stimulating factor, anti-thrombin III, interleukins, interferons, peptides A and C, fibrinogen, herceptin™, leptin, glycosidases, among many others. This list of peptides is exemplary and should not be considered to be exclusive. Rather, as is apparent from the disclosure provided herein, the methods of the invention are applicable to any peptide in which a desired glycan structure can be fashioned.

The methods of the invention are also useful for modifying chimeric peptides, including, but not limited to, chimeric peptides that include a moiety derived from an immunoglobulin, such as IgG.

Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for

enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a peptide creates a potential glycosylation site. As described elsewhere herein, O-linked glycosylation refers to the attachment of one sugar (*e.g.*, N-acetylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to a hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Several exemplary embodiments of the invention are discussed below. While several of these embodiments use peptides having names having trademarks, and other specific peptides as the exemplary peptide, these examples are not confined to any specific peptide.

The following exemplary embodiments are contemplated to include all peptide equivalents and variants of any peptide. Such variants include, but are not limited to, adding and deleting N-linked and O-linked glycosylation sites, and fusion proteins with added glycosylation sites. One of skill in the art will appreciate that the following embodiments and the basic methods disclosed therein can be applied to many peptides with equal success.

In one exemplary embodiment, the present invention provides methods for modifying Granulocyte Colony Stimulating Factor (G-CSF). Figures 29A to 29G set forth some examples of how this is accomplished using the methodology disclosed herein. In Figure 29B, a G-CSF peptide that is expressed in a mammalian cell system is trimmed back using a sialidase. The residues thus exposed are modified by the addition of a sialic acid-poly(ethylene glycol) moiety (PEG moiety), using an appropriate donor therefor and ST3Gal1. Figure 29C sets forth an exemplary scheme for modifying a G-CSF peptide that is expressed in an insect cell. The peptide is modified by adding a galactose moiety using an appropriate donor thereof and a galactosyltransferase. The galactose residues are functionalized with PEG via a sialic acid-PEG derivative, through the action of ST3Gal1. In Figure 29D, bacterially expressed G-CSF is contacted with an N-acetylgalactosamine donor and N-acetylgalactosamine transferase. The peptide is functionalized with PEG, using a PEGylated sialic acid donor and a sialyltransferase. In Figure 29E, mammalian cell expressed G-CSF is contacted with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue on the glycan on the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine-PEG. In Figure 29F, bacterially expressed G-CSF is remodeled by contacting the peptide

with an endo-GalNAc enzyme under conditions where it functions in a synthetic, rather than a hydrolytic manner, thereby adding a PEG-Gal-GalNAc molecule from an activated derivative thereof. Figure 29G provides another route for remodeling bacterially expressed G-CSF. The polypeptide is derivatized with a PEGylated N-acetylgalactosamine residue by  
5 contacting the polypeptide with an N-acetylgalactosamine transferase and an appropriate donor of PEGylated N-acetylgalactosamine.

In another exemplary embodiment, the invention provides methods for modifying Interferon  $\alpha$ -14C (IFN $\alpha$ 14C), as shown in Figures 30A to 30N. The various forms of IFN $\alpha$  are disclosed elsewhere herein. In Figure 30B, IFN $\alpha$ 14C expressed in mammalian cells is  
10 first treated with sialidase to trim back the sialic acid units thereon, and then the molecule is PEGylated using ST3Gal3 and a PEGylated sialic acid donor. In Figure 30C, N-acetylglucosamine is first added to IFN $\alpha$ 14C which has been expressed in insect or fungal cells, where the reaction is conducted via the action of GnT-I and/or II using an N-acetylglucosamine donor. The polypeptide is then PEGylated using a galactosyltransferase  
15 and a donor of PEG-galactose. In Figure 30D, IFN $\alpha$ 14C expressed in yeast is first treated with Endo-H to trim back the glycosyl units thereon. The molecule is galactosylated using a galactosyltransferase and a galactose donor, and it is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30F, IFN $\alpha$ 14C produced by mammalian cells is modified to include a PEG moiety using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30G,  
20 IFN $\alpha$ 14C expressed in insect or fungal cells first has N-acetylglucosamine added using one or more of GnT-I, II, IV, and V, and an N-acetylglucosamine donor. The protein is subsequently galactosylated using an appropriate donor and a galactosyltransferase. Then, IFN $\alpha$ 14C is PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30H, yeast produced IFN $\alpha$ 14C is first treated with mannosidases to trim back the mannosyl groups. N-  
25 acetylglucosamine is then added using a donor of N-acetylglucosamine and one or more of GnT-I, II, IV, and V. IFN $\alpha$ 14C is further galactosylated using an appropriate donor and a galactosyltransferase. Then, the polypeptide is PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30I, NSO cell expressed IFN $\alpha$ 14C is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid,  
30 thereby adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG.

In Figure 30J, IFN $\alpha$ 14C expressed by mammalian cells is PEGylated using a donor of PEG-sialic acid and  $\alpha$  2,8-sialyltransferase. In Figure 30K, IFN $\alpha$ 14C produced by mammalian cells is first treated with sialidase to trim back the terminal sialic acid residues, and then the molecule is PEGylated using trans-sialidase and PEGylated sialic acid-lactose complex. In Figure 30L, IFN $\alpha$ 14C expressed in a mammalian system is sialylated using a donor of sialic acid and  $\alpha$  2,8-sialyltransferase. In Figure 30M, IFN $\alpha$ 14C expressed in insect or fungal cells first has N-acetylglucosamine added using an appropriate donor and GnT-I and/or II. The molecule is then contacted with a galactosyltransferase and a galactose donor that is derivatized with a reactive sialic acid via a linker, so that the polypeptide is attached to the reactive sialic acid via the linker and the galactose residue. The polypeptide is then contacted with ST3Gal3 and transferrin, and thus becomes connected with transferrin via the sialic acid residue. In Figure 30N, IFN $\alpha$ 14C expressed in either insect or fungal cells is first treated with endoglycanase to trim back the glycosyl groups, and is then contacted with a galactosyltransferase and a galactose donor that is derivatized with a reactive sialic acid via a linker, so that the polypeptide is attached to the reactive sialic acid via the linker and the galactose residue. The molecule is then contacted with ST3Gal3 and transferrin, and thus becomes connected with transferrin via the sialic acid residue.

In another exemplary embodiment, the invention provides methods for modifying Interferon  $\alpha$ -2a or 2b (IFN $\alpha$ ), as shown in Figures 30O to 30EE. In Figure 30P, IFN $\alpha$  produced in mammalian cells is first treated with sialidase to trim back the glycosyl units, and is then PEGylated using ST3Gal3 and a PEGylated sialic acid donor. In Figure 30Q, IFN $\alpha$  expressed in insect cells is first galactosylated using an appropriate donor and a galactosyltransferase, and is then PEGylated using ST3Gal1 and a PEGylated sialic acid donor. Figure 30R offers another method for remodeling IFN $\alpha$  expressed in bacteria: PEGylated N-acetylgalactosamine is added to the protein using an appropriate donor and N-acetylgalactosamine transferase. In Figure 30S, IFN $\alpha$  expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine-PEG. In Figure 30T, IFN $\alpha$  expressed in bacteria is PEGylated using a modified enzyme Endo-N-acetylgalactosamidase, which functions in a synthetic instead of a hydrolytic

manner, and using a N-acetylgalactosamine donor derivatized with a PEG moiety. In Figure 30U, N-acetylgalactosamine is first added IFN $\alpha$  using an appropriate donor and N-acetylgalactosamine transferase, and then is PEGylated using a sialyltransferase and a PEGylated sialic acid donor. In Figure 30V, IFN $\alpha$  expressed in a mammalian system is first  
5 treated with sialidase to trim back the sialic acid residues, and is then PEGylated using a suitable donor and ST3Gal1 and/or ST3Gal3. In Figure 30W, IFN $\alpha$  expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues. The polypeptide is then contacted with ST3Gal1 and two reactive sialic acid residues that are connect via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and the  
10 second sialic acid residue. The polypeptide is subsequently contacted with ST3Gal3 and transferrin, and thus becomes connected with transferrin via the sialic acid residue. In Figure 30Y, IFN $\alpha$  expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, and is then PEGylated using ST3Gal1 and a donor of PEG-sialic acid. In Figure 30Z, IFN $\alpha$  produced by insect cells is PEGylated using a galactosyltransferase and a  
15 donor of PEGylated galactose. In Figure 30AA, bacterially expressed IFN $\alpha$  first has N-acetylgalactosamine added using a suitable donor and N-acetylgalactosamine transferase. The protein is then PEGylated using a sialyltransferase and a donor of PEG-sialic acid. In Figure 30CC, IFN $\alpha$  expressed in bacteria is modified in another procedure: PEGylated N-acetylgalactosamine is added to the protein by N-acetylgalactosamine transferase using a  
20 donor of PEGylated N-acetylgalactosamine. In Figure 30DD, IFN $\alpha$  expressed in bacteria is remodeled in yet another scheme. The polypeptide is first contacted with N-acetylgalactosamine transferase and a donor of N-acetylgalactosamine that is derivatized with a reactive sialic acid via a linker, so that IFN $\alpha$  is attached to the reactive sialic acid via the linker and the N-acetylgalactosamine. IFN $\alpha$  is then contacted with ST3Gal3 and asialo-  
25 transferrin so that it becomes connected with transferrin via the sialic acid residue. Then, IFN $\alpha$  is capped with sialic acid residues using ST3Gal3 and a sialic acid donor. An additional method for modifying bacterially expressed IFN $\alpha$  is disclosed in Figure 30EE, where IFN $\alpha$  is first exposed to NHS-CO-linker-SA-CMP and is then connected to a reactive sialic acid via the linker. It is subsequently conjugated with transferrin using ST3Gal3 and  
30 transferrin.

The methods for remodeling INN omega are essentially identical to those presented here for IFN alpha except that the attachment of the glycan to the IFN omega peptide occurs at amino acid residue 101 in SEQ ID NO:75. The nucleotide and amino acid sequences for IFN omega are presented herein as SEQ ID NOS:74 and 75. Methods of making and using IFN omega are found in U.S. Patent No. 4,917,887 and 5,317,089, and in EP Patent No. 0170204-A.

In another exemplary embodiment, the invention provides methods for modifying Interferon  $\beta$  (IFN- $\beta$ ), as shown in Figures 31A to 31S. In Figure 31B, IFN- $\beta$  expressed in a mammalian system is first treated with sialidase to trim back the terminal sialic acid residues.

The protein is then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. Figure 31C is a scheme for modifying IFN- $\beta$  produced by insect cells. First, N-acetylglucosamine is added to IFN- $\beta$  using an appropriate donor and GnT-I and/or -II. The protein is then galactosylated using a galactose donor and a galactosyltransferase. Finally, IFN- $\beta$  is PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 31D, IFN- $\beta$  expressed

in yeast is first treated with Endo-H to trim back its glycosyl chains, and is then galactosylated using a galactose donor and a galactosyltransferase, and is then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. In Figure 31E, IFN- $\beta$  produced by mammalian cells is modified by PEGylation using ST3Gal3 and a donor of sialic acid already derivatized with a PEG moiety. In Figure 31F, IFN- $\beta$  expressed in insect cells first has N-

acetylglucosamine added by one or more of GnT-I, II, IV, and V using a N-acetylglucosamine donor, and then is galactosylated using a galactose donor and a galactosyltransferase, and is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 31G, IFN- $\beta$  expressed in yeast is first treated with mannosidases to trim back the mannosyl units, then has N-acetylglucosamine added using a N-acetylglucosamine donor and

one or more of GnT-I, II, IV, and V. The protein is further galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a PEG-sialic acid donor. In Figure 31H, mammalian cell expressed IFN- $\beta$  is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide,

the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 31I, IFN- $\beta$  expressed in a mammalian system is PEGylated using a donor of PEG-sialic acid and  $\alpha$

2,8-sialyltransferase. In Figure 31J, IFN- $\beta$  expressed by mammalian cells is first treated with sialidase to trim back its terminal sialic acid residues, and then PEGylated using trans-sialidase and a donor of PEGylated sialic acid. In Figure 31K, IFN- $\beta$  expressed in mammalian cells is first treated with sialidase to trim back terminal sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then sialylated using ST3Gal3 and a sialic acid donor. In Figure 31L, IFN- $\beta$  expressed in mammalian cells is first treated with sialidase and galactosidase to trim back the glycosyl chains, then galactosylated using a galactose donor and an  $\alpha$ -galactosyltransferase, and then PEGylated using ST3Gal3 or a sialyltransferase and a donor of PEG-sialic acid. In Figure 31M, IFN- $\beta$  expressed in mammalian cells is first treated with sialidase to trim back the glycosyl units. It is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and is then sialylated using ST3Gal3 and a sialic acid donor. In Figure 31N, IFN- $\beta$  expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 31O, IFN- $\beta$  expressed in mammalian cells is sialylated using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 31Q, IFN- $\beta$  produced by insect cells first has N-acetylglucosamine added using a donor of N-acetylglucosamine and one or more of GnT-I, II, IV, and V, and is further PEGylated using a donor of PEG-galactose and a galactosyltransferase. In Figure 31R, IFN- $\beta$  expressed in yeast is first treated with endoglycanase to trim back the glycosyl groups, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 31S, IFN- $\beta$  expressed in a mammalian system is first contacted with ST3Gal3 and two reactive sialic acid residues connected via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and the second sialic acid residue. The polypeptide is then contacted with ST3Gal3 and desialylated transferrin, and thus becomes connected with transferrin via the sialic acid residue. Then, IFN- $\beta$  is further sialylated using a sialic acid donor and ST3Gal3.

In another exemplary embodiment, the invention provides methods for modifying Factor VII or VIIa, as shown in Figures 32 A to 32D. In Figure 32B, Factor VII or VIIa produced by a mammalian system is first treated with sialidase to trim back the terminal



sialic acid residues, and then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. Figure 32C, Factor VII or VIIa expressed by mammalian cells is first treated with sialidase to trim back the terminal sialic acid residues, and then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. Further, the polypeptide is sialylated with ST3Gal3 and a sialic acid donor. Figure 32D offers another modification scheme for Factor VII or VIIa produced by mammalian cells: the polypeptide is first treated with sialidase and galactosidase to trim back its sialic acid and galactose residues, then galactosylated using a galactosyltransferase and a galactose donor, and then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid.

In another exemplary embodiment, the invention provides methods for modifying Factor IX, some examples of which are included in Figures 33A to 33G. In Figure 33B, Factor IX produced by mammalian cells is first treated with sialidase to trim back the terminal sialic acid residues, and is then PEGylated with ST3Gal3 using a PEG-sialic acid donor. In Figure 33C, Factor IX expressed by mammalian cells is first treated with sialidase to trim back the terminal sialic acid residues, it is then PEGylated using ST3Gal3 and a PEG-sialic acid donor, and further sialylated using ST3Gal1 and a sialic acid donor. Another scheme for remodeling mammalian cell produced Factor IX can be found in Figure 33D. The polypeptide is first treated with sialidase to trim back the terminal sialic acid residues, then galactosylated using a galactose donor and a galactosyltransferase, further sialylated using a sialic acid donor and ST3Gal3, and then PEGylated using a donor of PEGylated sialic acid and ST3Gal1. In Figure 33E, Factor IX that is expressed in a mammalian system is PEGylated through the process of sialylation catalyzed by ST3Gal3 using a donor of PEG-sialic acid. In Figure 33F, Factor IX expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. Figure 33G provides an additional method of modifying Factor IX. The polypeptide, produced by mammalian cells, is PEGylated using a donor of PEG-sialic acid and  $\alpha$  2,8-sialyltransferase.

In another exemplary embodiment, the invention provides methods for modification of Follicle Stimulating Hormone (FSH). Figures 34A to 34J present some examples. In Figure 34B, FSH is expressed in a mammalian system and modified by treatment of sialidase to trim back terminal sialic acid residues, followed by PEGylation using ST3Gal3 and a

donor of PEG-sialic acid. In Figure 34C, FSH expressed in mammalian cells is first treated with sialidase to trim back terminal sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then sialylated using ST3Gal3 and a sialic acid donor. Figure 34D provides a scheme for modifying FSH expressed in a mammalian system. The

5 polypeptide is treated with sialidase and galactosidase to trim back its sialic acid and galactose residues, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 34E, FSH expressed in mammalian cells is modified in the following procedure: FSH is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a

10 donor of PEG-sialic acid, and is then sialylated using ST3Gal3 and a sialic acid donor.

Figure 34F offers another example of modifying FSH produced by mammalian cells: The polypeptide is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After

addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a  
15 hydrazine- or amine- PEG. In Figure 34G, FSH expressed in a mammalian system is modified in another procedure: the polypeptide is remodeled with addition of sialic acid using a sialic acid donor and an  $\alpha$  2,8-sialyltransferase. In Figure 34H, FSH is expressed in insect cells and modified in the following procedure: N-acetylglucosamine is first added to FSH using an appropriate N-acetylglucosamine donor and one or more of GnT-I, II, IV, and V;

20 FSH is then PEGylated using a donor of PEG-galactose and a galactosyltransferase. Figure 34I depicts a scheme of modifying FSH produced by yeast. According to this scheme, FSH is first treated with endoglycanase to trim back the glycosyl groups, galactosylated using a galactose donor and a galactosyltransferase, and is then PEGylated with ST3Gal3 and a donor of PEG-sialic acid. In Figure 34J, FSH expressed by mammalian cells is first contacted with  
25 ST3Gal3 and two reactive sialic acid residues via a linker, so that the polypeptide is attached to a reactive sialic acid via the linker and a second sialic acid residue. The polypeptide is then contacted with ST3Gal1 and desialylated chorionic gonadotrophin (CG) produced in CHO, and thus becomes connected with CG via the second sialic acid residue. Then, FSH is sialylated using a sialic acid donor and ST3Gal3 and/or ST3Gal1.

30 In another exemplary embodiment, the invention provides methods for modifying erythropoietin (EPO), Figures 35A to 35AA set forth some examples which are relevant to

the remodeling of both wild-type and mutant EPO peptides. In Figure 35B, EPO expressed in various mammalian systems is remodeled by contacting the expressed protein with a sialidase to remove terminal sialic acid residues. The resulting peptide is contacted with a sialyltransferase and a CMP-sialic acid that is derivatized with a PEG moiety. In Figure 35C, EPO that is expressed in insect cells is remodeled with N-acetylglucosamine, using GnT-I and/or GnT-II. Galactose is then added to the peptide, using galactosyltransferase. PEG group is added to the remodeled peptide by contacting it with a sialyltransferase and a CMP-sialic acid that is derivatized with a PEG moiety. In Figure 35D, EPO that is expressed in a mammalian cell system is remodeled by removing terminal sialic acid moieties via the action of a sialidase. The terminal galactose residues of the N-linked glycosyl units are "capped" with sialic acid, using ST3Gal3 and a sialic acid donor. The terminal galactose residues on the O-linked glycan are functionalized with a sialic acid bearing a PEG moiety, using an appropriate sialic acid donor and ST3Gal1. In Figure 35E, EPO that is expressed in a mammalian cell system is remodeled by functionalizing the N-linked glycosyl residues with a PEG-derivatized sialic acid moiety. The peptide is contacted with ST3Gal3 and an appropriately modified sialic acid donor. In Figure 35F, EPO that is expressed in an insect cell system, yeast or fungi, is remodeled by adding at least one N-acetylglucosamine residues by contacting the peptide with a N-acetylglucosamine donor and one or more of GnT-I, GnT-II, and GnT-V. The peptide is then PEGylated by contacting it with a PEGylated galactose donor and a galactosyltransferase. In Figure 35G, EPO that is expressed in an insect cell system, yeast or fungi, is remodeled by the addition of at least one N-acetylglucosamine residues, using an appropriate N-acetylglucosamine donor and one or more of GnT-I, GnT-II, and GnT-V. A galactosidase that is altered to operate in a synthetic, rather than a hydrolytic manner is used to add an activated PEGylated galactose donor to the N-acetylglucosamine residues. In Figure 35H, EPO that is expressed in an insect cell system, yeast or fungi, is remodeled by the addition of at least one terminal N-acetylglucosamine-PEG residue. The peptide is contacted with GnT-I and an appropriate N-acetylglucosamine donor that is derivatized with a PEG moiety. In Figure 35I, EPO that is expressed in an insect cell system, yeast or fungi, is remodeled by adding one or more terminal galactose-PEG residues. The peptide is contacted with GnT-I and an appropriate N-acetylglucosamine donor that is derivatized with a PEG moiety. The peptide is then contacted with galactosyltransferase and

an appropriate galactose donor that is modified with a PEG moiety. In Figure 35J, EPO expressed in an insect cell system, yeast or fungi, is remodeled by the addition of one more terminal sialic acid-PEG residues. The peptide is contacted with an appropriate N-acetylglucosamine donor and GnT-I. The peptide is further contacted with

5 galactosyltransferase and an appropriate galactose donor. The peptide is then contacted with ST3Gal3 and an appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35K, EPO expressed in an insect cell system, yeast or fungi, is remodeled by the addition of terminal sialic acid-PEG residues. The peptide is contacted with an appropriate N-acetylglucosamine donor and one or more of GnT-I, GnT-II, and GnT-V. The peptide is

10 then contacted with galactosyltransferase and an appropriate galactose donor. The peptide is further contacted with ST3Gal3 and an appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35L, EPO expressed in an insect cell system, yeast or fungi, is remodeled by the addition of one or more terminal  $\alpha$ 2,6-sialic acid-PEG residues. The peptide is contacted with an appropriate N-acetylglucosamine donor and one or more of GnT-

15 I, GnT-II, and GnT-V. The peptide is further contacted with galactosyltransferase and an appropriate galactose donor. The peptide is then contacted with  $\alpha$ 2,6-sialyltransferase and an appropriately modified sialic acid donor. In Figure 35M, EPO expressed in a mammalian cell system is remodeled by addition of one or more terminal sialic acid-PEG residues. The peptide is contacted with a sialidase to remove terminal sialic acid residues. The peptide is

20 further contacted with a sialyltransferase and an appropriate sialic acid donor. The peptide is further contacted with a sialyltransferase and an appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35N, EPO expressed in a mammalian cell system is remodeled by the addition of one or more terminal sialic acid-PEG residues. The peptide is contacted with a sialyltransferase and an appropriate sialic acid donor that is derivatized with

25 a PEG moiety. In Figure 35O, EPO expressed in a mammalian cell system is remodeled by the addition of one or more terminal  $\alpha$ 2,8-sialic acid-PEG residues to primarily O-linked glycans. The peptide is contacted with  $\alpha$ 2,8-sialyltransferase and an appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35P, EPO expressed in a mammalian cell is remodeled by the addition of one or more terminal  $\alpha$ 2,8-sialic acid-PEG residues to O-

30 linked and N-linked glycans. The peptide is contacted with  $\alpha$ 2,8-sialyltransferase and an

appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35Q, EPO expressed in yeast or fungi is remodeled by the addition of one or more terminal sialic acid-PEG residues. The peptide is contacted with mannosidases to remove terminal mannose residues. Next, the peptide is contacted with GnT-I<sup>2</sup> and an appropriate N-acetylglucosamine donor. The peptide is further contacted with galactosyltransferase and an appropriate galactose donor. The peptide is then contacted with a sialyltransferase and an appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35R, EPO expressed in yeast or fungi is remodeled by the addition of at least one terminal N-acetylglucosamine-PEG residues. The peptide is contacted with mannosidases to remove terminal mannose residue. The peptide is then contacted with GnT-I and an appropriate N-acetylglucosamine donor that is derivatized with a PEG moiety. In Figure 35S, EPO expressed in yeast or fungi is remodeled by the addition of one or more terminal sialic acid-PEG residues. The peptide is contacted with mannosidase-I to remove  $\alpha 2$  mannose residues. The peptide is further contacted with GnT-I and an appropriate N-acetylglucosamine donor. The peptide is then contacted with galactosyltransferase and an appropriate galactose donor. The peptide is then contacted with a sialyltransferase and an appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35U, EPO expressed in yeast or fungi is remodeled by addition of one or more galactose-PEG residues. The peptide is contacted with endo-H to trim back glycosyl groups. The peptide is then contacted with galactosyltransferase and an appropriate galactose donor that is derivatized with a PEG moiety. In Figure 35V, EPO expressed in yeast or fungi is remodeled by the addition of one or more terminal sialic acid-PEG residues. The peptide is contacted with endo-H to trim back glycosyl groups. The peptide is further contacted with galactosyltransferase and an appropriate galactose donor. The peptide is then contacted with a sialyltransferase and an appropriate sialic acid donor that is derivatized with a PEG moiety. In Figure 35W, EPO expressed in an insect cell system is remodeled by the addition of terminal galactose-PEG residues. The peptide is contacted with mannosidases to remove terminal mannose residues. The peptide is then contacted with galactosyltransferase and an appropriate galactose donor that is derivatized with a PEG moiety. In Figure 35Y, a mutant EPO called "novel erythropoiesis-stimulating protein" or NESP, expressed in NSO murine myeloma cells is remodeled by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid

donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine-PEG. In Figure 35Z, mutant EPO, i.e. NESP, expressed in a mammalian cell system is remodeled by addition of one or more terminal sialic acid-PEG residues. PEG is added to the glycosyl residue on the glycan using a PEG-modified sialic acid and an  $\alpha$  2,8-sialyltransferase. In Figure 35AA, NESP expressed in a mammalian cell system is remodeled by the addition of terminal sialic acid residues. The sialic acid is added to the glycosyl residue using a sialic acid donor and an  $\alpha$ 2,8-sialyltransferase.

In another exemplary embodiment, the invention provides methods for modifying granulocyte-macrophage colony-stimulating factor (GM-CSF), as shown in Figures 36A to 36K. In Figure 36B, GM-CSF expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 36C, GM-CSF expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then is further sialylated using a sialic acid donor and ST3Gal1 and/or ST3Gal3. In Figure 36D, GM-CSF expressed in NSO cells is first treated with sialidase and  $\alpha$ -galactosidase to trim back the glycosyl groups, then sialylated using a sialic acid donor and ST3Gal3, and is then PEGylated using ST3Gal1 and a donor of PEG-sialic acid. In Figure 36E, GM-CSF expressed in mammalian cells is first treated with sialidase to trim back sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then is further sialylated using ST3Gal3 and a sialic acid donor. In Figure 36F, GM-CSF expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 36G, GM-CSF expressed in mammalian cells is sialylated using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 36I, GM-CSF expressed in insect cells is modified by addition of N-acetylglucosamine using a suitable donor and one or more of GnT-I, II, IV, and V, followed by addition of PEGylated galactose using a suitable donor and a galactosyltransferase. In Figure 36J, yeast expressed GM-CSF is first treated with endoglycanase and/or mannosidase to trim back the glycosyl units, and subsequently PEGylated using a galactosyltransferase and a donor of PEG-galactose. In

Figure 36K, GM-CSF expressed in mammalian cells is first treated with sialidase to trim back sialic acid residues, and is subsequently sialylated using ST3Gal3 and a sialic acid donor. The polypeptide is then contacted with ST3Gal1 and two reactive sialic acid residues connected via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and second sialic acid residue. The polypeptide is further contacted with ST3Gal3 and transferrin, and thus becomes connected with transferrin.

In another exemplary embodiment, the invention provides methods for modification of Interferon gamma (IFN $\gamma$ ). Figures 37A to 37N contain some examples. In Figure 37B, IFN $\gamma$  expressed in a variety of mammalian cells is first treated with sialidase to trim back terminal sialic acid residues, and is subsequently PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 37C, IFN $\gamma$  expressed in a mammalian system is first treated with sialidase to trim back terminal sialic acid residues. The polypeptide is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and is further sialylated with ST3Gal3 and a donor of sialic acid. In Figure 37D, mammalian cell expressed IFN $\gamma$  is first treated with sialidase and  $\alpha$ -galactosidase to trim back sialic acid and galactose residues. The polypeptide is then galactosylated using a galactose donor and a galactosyltransferase. Then, IFN $\gamma$  is PEGylated using a donor of PEG-sialic acid and ST3Gal3. In Figure 37E, IFN $\gamma$  that is expressed in a mammalian system is first treated with sialidase to trim back terminal sialic acid residues. The polypeptide is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and is further sialylated with ST3Gal3 and a sialic acid donor. Figure 37F describes another method for modifying IFN $\gamma$  expressed in a mammalian system. The protein is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 37G, IFN $\gamma$  expressed in mammalian cells is remodeled by addition of sialic acid using a sialic acid donor and an  $\alpha$  2,8-sialyltransferase. In Figure 37I, IFN $\gamma$  expressed in insect or fungal cells is modified by addition of N-acetylglucosamine using an appropriate donor and one or more of GnT-I, II, IV, and V. The protein is further modified by addition of PEG moieties using a donor of PEGylated galactose and a galactosyltransferase. Figure 37J offers a method for modifying IFN $\gamma$  expressed in yeast. The polypeptide is first treated with endoglycanase to trim back the saccharide chains, and then galactosylated using a galactose

donor and a galactosyltransferase. Then, IFN $\gamma$  is PEGylated using a donor of PEGylated sialic acid and ST3Gal3. In Figure 37K, IFN $\gamma$  produced by mammalian cells is modified as follows: the polypeptide is first contacted with ST3Gal3 and a donor of sialic acid that is derivatized with a reactive galactose via a linker, so that the polypeptide is attached to the reactive galactose via the linker and sialic acid residue. The polypeptide is then contacted with a galactosyltransferase and transferrin pre-treated with endoglycanase, and thus becomes connected with transferrin via the galactose residue. In the scheme illustrated by Figure 37L, IFN $\gamma$ , which is expressed in a mammalian system, is modified via the action of ST3Gal3: PEGylated sialic acid is transferred from a suitable donor to IFN $\gamma$ . Figure 37M is an example of modifying IFN $\gamma$  expressed in insect or fungal cells, where PEGylation of the polypeptide is achieved by transferring PEGylated N-acetylglucosamine from a donor to IFN $\gamma$  using GnT-I and/or II. In Figure 37N, IFN $\gamma$  expressed in a mammalian system is remodeled with addition of PEGylated sialic acid using a suitable donor and an  $\alpha$  2,8-sialyltransferase.

In another exemplary embodiment, the invention provides methods for modifying  $\alpha_1$  anti-trypsin ( $\alpha_1$ -protease inhibitor). Some such examples can be found in Figures 38A to 38N. In Figure 38B,  $\alpha_1$  anti-trypsin expressed in a variety of mammalian cells is first treated with sialidase to trim back sialic acid residues. PEGylated sialic acid residues are then added using an appropriate donor, such as CMP-SA-PEG, and a sialyltransferase, such as ST3Gal3. Figure 38C demonstrates another scheme of  $\alpha_1$  anti-trypsin modification.  $\alpha_1$  anti-trypsin expressed in a mammalian system is first treated with sialidase to trim back sialic acid residues. Sialic acid residues derivatized with PEG are then added using an appropriate donor and a sialyltransferase, such as ST3Gal3. Subsequently, the molecule is further modified by the addition of sialic acid residues using a sialic acid donor and ST3Gal3. Optionally, mammalian cell expressed  $\alpha_1$  anti-trypsin is first treated with sialidase and  $\alpha$ -galactosidase to trim back terminal sialic acid and  $\alpha$ -linkage galactose residues. The polypeptide is then galactosylated using galactosyltransferase and a suitable galactose donor. Further, sialic acid derivatized with PEG is added by the action of ST3Gal3 using a PEGylated sialic acid donor. In Figure 38D,  $\alpha_1$  anti-trypsin expressed in a mammalian system first has the terminal sialic acid residues trimmed back using sialidase. PEG is then added to N-linked glycosyl residues via the action of ST3Gal3, which mediates the transfer of PEGylated sialic acid from a donor, such as CMP-SA-PEG, to  $\alpha_1$  anti-trypsin. More sialic



acid residues are subsequently attached using a sialic acid donor and ST3Gal3. Figure 38E illustrates another process through which  $\alpha_1$  anti-trypsin is remodeled.  $\alpha_1$  anti-trypsin expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 38F, yet another method of  $\alpha_1$  anti-trypsin modification is disclosed.  $\alpha_1$  anti-trypsin obtained from a mammalian expression system is remodeled with addition of sialic acid using a sialic acid donor and an  $\alpha$  2,8-sialyltransferase. In Figure 38H,  $\alpha_1$  anti-trypsin is expressed in insect or yeast cells, and remodeled by the addition of terminal N-acetylglucosamine residues by way of contacting the polypeptide with UDP-N-acetylglucosamine and one or more of GnT-I, II, IV, or V. Then, the polypeptide is modified with PEG moieties using a donor of PEGylated galactose and a galactosyltransferase. In Figure 38I,  $\alpha_1$  anti-trypsin expressed in yeast cells is treated first with endoglycanase to trim back glycosyl chains. It is then galactosylated with a galactosyltransferase and a galactose donor. Then, the polypeptide is PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 38J,  $\alpha_1$  anti-trypsin is expressed in a mammalian system. The polypeptide is first contacted with ST3Gal3 and a donor of sialic acid that is derivatized with a reactive galactose via a linker, so that the polypeptide is attached to the reactive galactose via the linker and sialic acid residue. The polypeptide is then contacted with a galactosyltransferase and transferrin pre-treated with endoglycanase, and thus becomes connected with transferrin via the galactose residue. In Figure 38L,  $\alpha_1$  anti-trypsin expressed in yeast is first treated with endoglycanase to trim back its glycosyl groups. The protein is then PEGylated using a galactosyltransferase and a donor of galactose with a PEG moiety. In Figure 38M,  $\alpha_1$  anti-trypsin expressed in plant cells is treated with hexosaminidase, mannosidase, and xylosidase to trim back its glycosyl chains, and subsequently modified with N-acetylglucosamine derivatized with a PEG moiety, using N-acetylglucosamine transferase and a suitable donor. In Figure 38N,  $\alpha_1$  anti-trypsin expressed in mammalian cells is modified by adding PEGylated sialic acid residues using ST3Gal3 and a donor of sialic acid derivatized with PEG.

In another exemplary embodiment, the invention provides methods for modifying glucocerebrosidase ( $\beta$ -glucosidase, Cerezyme<sup>™</sup> or Ceredase<sup>™</sup>), as shown in Figures 39A to

39K. In Figure 39B, Cerezyme™ expressed in a mammalian system is first treated with sialidase to trim back terminal sialic acid residues, and is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 39C, Cerezyme™ expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then has mannose-6-phosphate group attached using ST3Gal3 and a reactive sialic acid derivatized with mannose-6-phosphate, and then is sialylated using ST3Gal3 and a sialic acid donor. Optionally, NSO cell expressed Cerezyme™ is first treated with sialidase and galactosidase to trim back the glycosyl groups, and is then galactosylated using a galactose donor and an  $\alpha$ -galactosyltransferase. Then, mannose-6-phosphate moiety is added to the molecule using ST3Gal3 and a reactive sialic acid derivatized with mannose-6-phosphate. In Figure 39D, Cerezyme™ expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, it is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and is then sialylated using ST3Gal3 and a sialic acid donor. In Figure 39E, Cerezyme™ expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as one or more mannose-6-phosphate groups. In Figure 39F, Cerezyme™ expressed in mammalian cells is sialylated using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 39H, Cerezyme™ expressed in insect cells first has N-acetylglucosamine added using a suitable donor and one or more of GnT-I, II, IV, and V, and then is PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 39I, Cerezyme™ expressed in yeast is first treated with endoglycanase to trim back the glycosyl groups, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 39JK, Cerezyme™ expressed in mammalian cells is first contacted with ST3Gal3 and two reactive sialic acid residues connected via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and the second sialic acid residue. The polypeptide is then contacted with ST3Gal3 and desialylated transferrin, and thus becomes connected with transferrin. Then, the polypeptide is sialylated using a sialic acid donor and ST3Gal3.

In another exemplary embodiment, the invention provides methods for modifying Tissue-Type Plasminogen Activator (TPA) and its mutant. Several specific modification

schemes are presented in Figures 40A to 40W. Figure 40B illustrates one modification procedure: after TPA is expressed by mammalian cells, it is treated with one or more of mannosidase(s) and sialidase to trim back mannosyl and/or sialic acid residues. Terminal N-acetylglucosamine is then added by contacting the polypeptide with a suitable donor of N-acetylglucosamine and one or more of GnT-I, II, IV, and V. TPA is further galactosylated using a galactose donor and a galactosyltransferase. Then, PEG is attached to the molecule by way of sialylation catalyzed by ST3Gal3 and using a donor of sialic acid derivatized with a PEG moiety. In Figure 40C, TPA is expressed in insect or fungal cells. The modification includes the steps of addition of N-acetylglucosamine using an appropriate donor of N-acetylglucosamine and GnT-I and/or II; galactosylation using a galactose donor and a galactosyltransferase; and attachment of PEG by way of sialylation using ST3Gal3 and a donor of sialic acid derivatized with PEG. In Figure 40D, TPA is expressed in yeast and subsequently treated with endoglycanase to trim back the saccharide chains. The polypeptide is further PEGylated via the action of a galactosyltransferase, which catalyzes the transfer of a PEG-galactose from a donor to TPA. In Figure 40E, TPA is expressed in insect or yeast cells. The polypeptide is then treated with  $\alpha$ - and  $\beta$ -mannosidases to trim back terminal mannosyl residues. Further, PEG moieties are attached to the molecule via transfer of PEG-galactose from a suitable donor to TPA, which is mediated by a galactosyltransferase. Figure 40F provides a different method for modification of TPA obtained from an insect or yeast system: the polypeptide is remodeled by addition of N-acetylglucosamine using a donor of N-acetylglucosamine and GnT-I and/or II, followed by PEGylation using a galactosyltransferase and a donor of PEGylated galactose. Figure 40G offers another scheme for remodeling TPA expressed in insect or yeast cells. Terminal N-acetylglucosamine is added using a donor of N-acetylglucosamine and GnT-I and/or II. A galactosidase that is modified to operate in a synthetic, rather than a hydrolytic manner, is utilized to add PEGylated galactose from a proper donor to the N-acetylglucosamine residues. In Figure 40I, TPA expressed in a mammalian system is first treated with sialidase and galactosidase to trim back sialic acid and galactose residues. The polypeptide is further modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 40J, TPA, which is

expressed in a mammalian system, is remodeled following this scheme: first, the polypeptide is treated with  $\alpha$ - and  $\beta$ -mannosidases to trim back the terminal mannosyl residues; sialic acid residues are then attached to terminal galactosyl residues using a sialic acid donor and ST3Gal3; further, TPA is PEGylated via the transfer of PEGylated galactose from a donor to a N-acetylglucosaminyl residue catalyzed by a galactosyltransferase. In Figure 40K, TPA is expressed in a plant system. The modification procedure in this example is as follows: TPA is first treated with hexosaminidase, mannosidase, and xylosidase to trim back its glycosyl groups; PEGylated N-acetylglucosamine is then added to TPA using a proper donor and N-acetylglucosamine transferase. In Figure 40M, a TPA mutant (TNK TPA), expressed in mammalian cells, is remodeled. Terminal sialic acid residues are first trimmed back using sialidase; ST3Gal3 is then used to transfer PEGylated sialic acid from a donor to TNK TPA, such that the polypeptide is PEGylated. In Figure 40N, TNK TPA expressed in a mammalian system is first treated with sialidase to trim back terminal sialic acid residues. The protein is then PEGylated using CMP-SA-PEG as a donor and ST3Gal3, and further sialylated using a sialic acid donor and ST3Gal3. In Figure 40O, NSO cell expressed TNK TPA is first treated with sialidase and  $\alpha$ -galactosidase to trim back terminal sialic acid and galactose residues. TNK TPA is then galactosylated using a galactose donor and a galactosyltransferase. The last step in this remodeling scheme is transfer of sialic acid derivatized with PEG moiety from a donor to TNK TPA using a sialyltransferase such as ST3Gal3. In Figure 40Q, TNK TPA is expressed in a mammalian system and is first treated with sialidase to trim back terminal sialic acid residues. The protein is then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. Then, the protein is sialylated using a sialic acid donor and ST3Gal3. In Figure 40R, TNK TPA expressed in a mammalian system is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 40S, TNK TPA expressed in mammalian cells is modified via a different method: the polypeptide is remodeled with addition of sialic acid using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 40U, TNK TPA expressed in insect cells is remodeled by addition of N-acetylglucosamine using an appropriate donor and one or more of GnT-I, II, IV, and V. The protein is further modified by addition of PEG moieties using a donor of

PEGylated galactose and a galactosyltransferase. In Figure 40V, TNK TPA is expressed in yeast. The polypeptide is first treated with endoglycanase to trim back its glycosyl chains and then PEGylated using a galactose donor derivatized with PEG and a galactosyltransferase. In Figure 40W, TNK TPA is produced in a mammalian system. The polypeptide is first contacted with ST3Gal3 and a donor of sialic acid that is derivatized with a reactive galactose via a linker, so that the polypeptide is attached to the reactive galactose via the linker and sialic acid residue. The polypeptide is then contacted with a galactosyltransferase and anti-TNF IG chimera produced in CHO, and thus becomes connected with the chimera via the galactose residue.

In another exemplary embodiment, the invention provides methods for modifying Interleukin-2 (IL-2). Figures 41A to 41G provide some examples. Figure 41B provides a two-step modification scheme: IL-2 produced by mammalian cells is first treated with sialidase to trim back its terminal sialic acid residues, and is then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. In Figure 41C, insect cell expressed IL-2 is modified first by galactosylation using a galactose donor and a galactosyltransferase. Subsequently, IL-2 is PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. In Figure 41D, IL-2 expressed in bacteria is modified with N-acetylgalactosamine using a proper donor and N-acetylgalactosamine transferase, followed by a step of PEGylation with a PEG-sialic acid donor and a sialyltransferase. Figure 41E offers another scheme of modifying IL-2 produced by a mammalian system. The polypeptide is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. Figure 41F illustrates an example of remodeling IL-2 expressed by *E. coli*. The polypeptide is PEGylated using a reactive N-acetylgalactosamine complex derivatized with a PEG group and an enzyme that is modified so that it functions as a synthetic enzyme rather than a hydrolytic one. In Figure 41G, IL-2 expressed by bacteria is modified by addition of PEGylated N-acetylgalactosamine using a proper donor and N-acetylgalactosamine transferase.

In another exemplary embodiment, the invention provides methods for modifying Factor VIII, as shown in Figures 42A to 42N. In Figure 42B, Factor VIII expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, and is then

PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 42C, Factor VIII expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a proper donor, and is then further sialylated using ST3Gal1 and a sialic acid donor.

5 In Figure 42E, mammalian cell produced Factor VIII is modified by the single step of PEGylation, using ST3Gal3 and a donor of PEGylated sialic acid. Figure 42F offers another example of modification of Factor VIII that is expressed by mammalian cells. The protein is PEGylated using ST3Gal1 and a donor of PEGylated sialic acid. In Figure 42G, mammalian cell expressed Factor VIII is remodeled following another scheme: it is PEGylated using  $\alpha$  2,8-sialyltransferase and a donor of PEG-sialic acid. In Figure 42 I, Factor VIII produce by mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 42J, Factor VIII expressed by mammalian cells is first treated with Endo-H to trim back glycosyl groups. It is then PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 42K, Factor VIII expressed in a mammalian system is first sialylated using ST3Gal3 and a sialic acid donor, then treated with Endo-H to trim back the glycosyl groups, and then PEGylated with a galactosyltransferase and a donor of PEG-galactose. In Figure 42L, Factor VIII expressed in a mammalian system is first treated with mannosidases to trim back terminal mannosyl residues, then has an N-acetylglucosamine group added using a suitable donor and GnT-I and/or II, and then is PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 42M, Factor VIII expressed in mammalian cells is first treated with mannosidases to trim back mannosyl units, then has N-acetylglucosamine group added using N-acetylglucosamine transferase and a suitable donor. It is further galactosylated using a galactosyltransferase and a galactose donor, and then sialylated using ST3Gal3 and a sialic acid donor. In Figure 42N, Factor VIII is produced by mammalian cells and modified as follows: it is first treated with mannosidases to trim back the terminal mannosyl groups. A PEGylated N-acetylglucosamine group is then added using GnT-I and a suitable donor of PEGylated N-acetylglucosamine.

In another exemplary embodiment, the invention provides methods for modifying urokinase, as shown in Figures 43A to 43M. In Figure 43B, urokinase expressed in mammalian cells is first treated with sialidase to trim back sialic acid residues, and is then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. In Figure 43C, urokinase expressed in mammalian cells is first treated with sialidase to trim back sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid, and then sialylated using ST3Gal3 and a sialic acid donor. Optionally, urokinase expressed in a mammalian system is first treated with sialidase and galactosidase to trim back glycosyl chains, then galactosylated using a galactose donor and an  $\alpha$ -galactosyltransferase, and then PEGylated using ST3Gal3 or sialyltransferase and a donor of PEG-sialic acid. In Figure 43D, urokinase expressed in mammalian cells is first treated with sialidase to trim back sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then further sialylated using ST3Gal3 and a sialic acid donor. In Figure 43E, urokinase expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 43F, urokinase expressed in mammalian cells is sialylated using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 43H, urokinase expressed in insect cells is modified in the following steps: first, N-acetylglucosamine is added to the polypeptide using a suitable donor of N-acetylglucosamine and one or more of GnT-I, II, IV, and V; then PEGylated galactose is added, using a galactosyltransferase and a donor of PEG-galactose. In Figure 43I, urokinase expressed in yeast is first treated with endoglycanase to trim back glycosyl groups, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 43J, urokinase expressed in mammalian cells is first contacted with ST3Gal3 and two reactive sialic acid residues that are connected via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and second sialic acid residue. The polypeptide is then contacted with ST3Gal1 and desialylated urokinase produced in mammalian cells, and thus becomes connected with a second molecule of urokinase. Then, the whole molecule is further sialylated using a sialic donor and ST3Gal1 and/or ST3Gal3. In Figure 43K, isolated urokinase is first treated with sulfohydrolase to remove sulfate

groups, and is then PEGylated using a sialyltransferase and a donor of PEG-sialic acid. In Figure 43LM, isolated urokinase is first treated with sulfohydrolase and hexosaminidase to remove sulfate groups and hexosamine groups, and then PEGylated using a galactosyltransferase and a donor of PEG-galactose.

5 In another exemplary embodiment, the invention provides methods for modifying DNase I, as shown in Figures 44A to 44J. In Figure 44B, DNase I is expressed in a mammalian system and modified in the following steps: first, the protein is treated with sialidase to trim back the sialic acid residues; then the protein is PEGylated with ST3Gal3 using a donor of PEG-sialic acid. In Figure 44C, DNase I expressed in mammalian cells is  
10 first treated with sialidase to trim back the sialic acid residues, then PEGylated with ST3Gal3 using a PEG-sialic acid donor, and is then sialylated using ST3Gal3 and a sialic acid donor. Optionally, DNase I expressed in a mammalian system is first exposed to sialidase and galactosidase to trim back the glycosyl groups, then galactosylated using a galactose donor and an  $\alpha$ -galactosyltransferase, and then PEGylated using ST3Gal3 or sialyltransferase and a  
15 donor of PEG-sialic acid. In Figure 44D, DNase I expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a PEG-sialic acid donor, and then sialylated with ST3Gal3 using a sialic acid donor. In Figure 44E, DNase I expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a  
20 reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 44F, DNase I expressed in mammalian cells is sialylated using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 44H, DNase I expressed in insect cells first has N-acetylglucosamine added using a suitable donor and one or more of GnT-I, II, IV, and V.  
25 The protein is then PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 44I, DNase I expressed in yeast is first treated with endoglycanase to trim back the glycosyl units, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 44JK, DNase I expressed in mammalian cells is first contacted with ST3Gal3 and two reactive sialic acid  
30 residues connected via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and the second sialic acid residue. The polypeptide is then contacted with



ST3Gal1 and desialylated  $\alpha$ -1-protease inhibitor, and thus becomes connected with the inhibitor via the sialic acid residue. Then, the polypeptide is further sialylated using a suitable donor and ST3Gal1 and/or ST3Gal3.

In another exemplary embodiment, the invention provides methods for modifying insulin that is mutated to contain an N-glycosylation site, as shown in Figures 45A to 45L. In Figure 45B, insulin expressed in a mammalian system is first treated with sialidase to trim back the sialic acid residues, and then PEGylated using ST3Gal3 and a PEG-sialic acid donor. In Figure 45C, insulin expressed in insect cells is modified by addition of PEGylated N-acetylglucosamine using an appropriate donor and GnT-I and/or II. In Figure 45D, insulin expressed in yeast is first treated with Endo-H to trim back the glycosyl groups, and then PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 45F, insulin expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues and then PEGylated using ST3Gal1 and a donor of PEG-sialic acid. In Figure 45G, insulin expressed in insect cells is modified by means of addition of PEGylated galactose using a suitable donor and a galactosyltransferase. In Figure 45H, insulin expressed in bacteria first has N-acetylgalactosamine added using a proper donor and N-acetylgalactosamine transferase. The polypeptide is then PEGylated using a sialyltransferase and a donor of PEG-sialic acid. In Figure 45J, insulin expressed in bacteria is modified through a different method: PEGylated N-acetylgalactosamine is added to the protein using a suitable donor and N-acetylgalactosamine transferase. In Figure 45K, insulin expressed in bacteria is modified following another scheme: the polypeptide is first contacted with N-acetylgalactosamine transferase and a reactive N-acetylgalactosamine that is derivatized with a reactive sialic acid via a linker, so that the polypeptide is attached to the reactive sialic acid via the linker and N-acetylgalactosamine. The polypeptide is then contacted with ST3Gal3 and asialo-transferrin, and therefore becomes connected with transferrin. Then, the polypeptide is sialylated using ST3Gal3 and a sialic acid donor. In Figure 45L, insulin expressed in bacteria is modified using yet another method: the polypeptide is first exposed to NHS-CO-linker-SA-CMP and becomes connected to the reactive sialic acid residue via the linker. The polypeptide is then conjugated to transferrin using ST3Gal3 and asialo-transferrin. Then, the polypeptide is further sialylated using ST3Gal3 and a sialic acid donor.

In another exemplary embodiment, the invention provides methods for modifying Hepatitis B antigen (M antigen-preS2 and S), as shown in Figures 46A to 46K. In Figure 46B, M-antigen is expressed in a mammalian system and modified by initial treatment of sialidase to trim back the sialic acid residues and subsequent conjugation with lipid A, using ST3Gal3 and a reactive sialic acid linked to lipid A via a linker. In Figure 46C, M-antigen expressed in mammalian cells is first treated with sialidase to trim back the terminal sialic acid residues, then conjugated with tetanus toxin via a linker using ST3Gal1 and a reactive sialic acid residue linked to the toxin via the linker, and then sialylated using ST3Gal3 and a sialic acid donor. In Figure 46D, M-antigen expressed in a mammalian system is first treated with a galactosidase to trim back galactosyl residues, and then sialylated using ST3Gal3 and a sialic acid donor. The polypeptide then has sialic acid derivatized with KLH added using ST3Gal1 and a suitable donor. In Figure 46E, yeast expressed M-antigen is first treated with a mannosidase to trim back the mannosyl residues, and then conjugated to a diphtheria toxin using GnT-I and a donor of N-acetylglucosamine linked to the diphtheria toxin. In Figure 46F, mammalian cell expressed M-antigen is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 46G, M-antigen obtained from a mammalian system is remodeled by sialylation using a sialic acid donor and poly  $\alpha$  2,8-sialyltransferase. In Figure 46I, M-antigen expressed in insect cells is conjugated to a Neisseria protein by using GnT-II and a suitable donor of N-acetylglucosamine linked to the Neisseria protein. In Figure 46J, yeast expressed M-antigen is first treated with endoglycanase to trim back its glycosyl chains, and then conjugated to a Neisseria protein using a galactosyltransferase and a proper donor of galactose linked to the Neisseria protein. Figure 46K is another example of modification of M-antigen expressed in yeast. The polypeptide is first treated with mannosidases to trim back terminal mannosyl residues, and then has N-acetylglucosamine added using GnT-I and/or II. Subsequently, the polypeptide is galactosylated using a galactose donor and a galactosyltransferase, and then capped with sialic acid residues using a sialyltransferase and a sialic acid donor.

In another exemplary embodiment, the invention provides methods for modifying human growth hormone (N, V, and variants thereof), as shown in Figures 47A to 47K. In

Figure 47B, human growth hormone either mutated to contain a N-linked site, or a naturally occurring isoform that has an N-linked side (i.e., the placental enzyme) produced by mammalian cells is first treated with sialidase to trim back terminal sialic acid residues and subsequently PEGylated with ST3Gal3 and using a donor of PEGylated sialic acid. In Figure 5 47C, human growth hormone expressed in insect cells is modified by addition of PEGylated N-acetylglucosamine using GnT-I and/or II and a proper donor of PEGylated N-acetylglucosamine. In Figure 47D, human growth hormone is expressed in yeast, treated with Endo-H to trim back glycosyl groups, and further PEGylated with a galactosyltransferase using a donor of PEGylated galactose. In Figure 47F, human growth hormone-mucin fusion protein expressed in a mammalian system is modified by initial treatment of sialidase to trim back sialic acid residues and subsequent PEGylation using a donor of PEG-sialic acid and ST3Gal1. In Figure 47G, human growth hormone-mucin fusion protein expressed in insect cells is remodeled by PEGylation with a galactosyltransferase and using a donor of PEGylated galactose. In Figure 47H, human growth hormone-mucin fusion protein is produced in bacteria. N-acetylgalactosamine is first added to the fusion protein by the action of N-acetylgalactosamine transferase using a donor of N-acetylgalactosamine, followed by PEGylation of the fusion protein using a donor of PEG-sialic acid and a sialyltransferase. Figure 47I describes another scheme of modifying bacterially expressed human growth hormone-mucin fusion protein: the fusion protein is PEGylated through the action of N-acetylgalactosamine transferase using a donor of PEGylated N- 20 acetylgalactosamine. Figure 47J provides a further remodeling scheme for human growth hormone-mucin fusion protein. The fusion protein is first contacted with N-acetylgalactosamine transferase and a donor of N-acetylgalactosamine that is derivatized with a reactive sialic acid via a linker, so that the fusion protein is attached to the reactive sialic acid via the linker and N-acetylgalactosamine. The fusion protein is then contacted with a sialyltransferase and asialo-transferrin, and thus becomes connected with transferrin via the sialic acid residue. Then, the fusion protein is capped with sialic acid residues using ST3Gal3 and a sialic acid donor. In Figure 47K, yet another scheme is given for modification of human growth hormone(N) produced in bacteria. The polypeptide is first 25 contacted with NHS-CO-linker-SA-CMP and becomes coupled with the reactive sialic acid through the linker. The polypeptide is then contacted with ST3Gal3 and asialo-transferrin

and becomes linked to transferrin via the sialic acid residue. Then, the polypeptide is sialylated using ST3Gal3 and a sialic acid donor.

In another exemplary embodiment, the invention provides methods for remodeling TNF receptor IgG fusion protein (TNFR-IgG, or Enbrel™), as shown in Figures 48A to 48G.

- 5 Figure 48B illustrates a modification procedure in which TNFR-IgG, expressed in a mammalian system is first sialylated with a sialic acid donor and a sialyltransferase, ST3Gal1; the fusion protein is then galactosylated with a galactose donor and a galactosyltransferase; then, the fusion protein is PEGylated via the action of ST3Gal3 and a donor of sialic acid derivatized with PEG. In Figure 48C, TNFR-IgG expressed in
- 10 mammalian cells is initially treated with sialidase to trim back sialic acid residues. PEG moieties are subsequently attached to TNFR-IgG by way of transferring PEGylated sialic acid from a donor to the fusion protein in a reaction catalyzed by ST3Gal1. In Figure 48D, TNFR-IgG is expressed in a mammalian system and modified by addition of PEG through the galactosylation process, which is mediated by a galactosyltransferase using a PEG-
- 15 galactose donor. In Figure 48E, TNFR-IgG is expressed in a mammalian system. The first step in remodeling of the fusion protein is adding O-linked sialic acid residues using a sialic acid donor and a sialyltransferase, ST3Gal1. Subsequently, PEGylated galactose is added to the fusion protein using a galactosyltransferase and a suitable donor of galactose with a PEG moiety. In Figure 48F, TNFR-IgG expressed in mammalian cells is modified first by capping
- 20 appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the fusion protein, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 48G, TNFR-IgG expressed in mammalian cells is remodeled by 2,8-
- 25 sialyltransferase, which catalyzes the reaction in which PEGylated sialic acid is transferred to the fusion protein from a donor of sialic acid with a PEG moiety.

- In another exemplary embodiment, the invention provides methods for generating Herceptin™ conjugates, as shown in Figures 49A to 49D. In Figure 49B, Herceptin™ is expressed in a mammalian system and is first galactosylated using a galactose donor and a galactosyltransferase. Herceptin™ is then conjugated with a toxin via a sialic acid through
- 30 the action of ST3Gal3 using a reactive sialic acid-toxin complex. In Figure 49C, Herceptin™ produced in either mammalian cells or fungi is conjugated to a toxin through the process of

galactosylation, using a galactosyltransferase and a reactive galactose-toxin complex. Figure 49D contains another scheme of making Herceptin™ conjugates: Herceptin™ produced in fungi is first treated with Endo-H to trim back glycosyl groups, then galactosylated using a galactose donor and a galactosyltransferase, and then conjugated with a radioisotope by way of sialylation, by using ST3Gal3 and a reactive sialic acid-radioisotope complex. Alternatively, the reactive sialic acid moiety may have attached only the chelating moiety can then be loaded with radioisotope at a subsequent stage.

In another exemplary embodiment, the invention provides methods for making Synagis™ conjugates, as shown in Figures 50A to 50D. In Figure 50B, Synagis™ expressed in mammalian cells is first galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 50C, Synagis™ expressed in mammalian or fungal cells is PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 50D, Synagis™ expressed in first treated with Endo-H to trim back the glycosyl groups, then galactosylated using a galactose donor and a galactosyltransferase, and is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid.

In another exemplary embodiment, the invention provides methods for generating Remicade™ conjugates, as shown in Figures 51A to 51D. In Figure 51B, Remicade™ expressed in a mammalian system is first galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 51C, Remicade™ expressed in a mammalian system is modified by addition of PEGylated galactose using a suitable donor and a galactosyltransferase. In Figure 51D, Remicade™ expressed in fungi is first treated with Endo-H to trim back the glycosyl chains, then galactosylated using a galactose donor and a galactosyltransferase, and then conjugated to a radioisotope using ST3Gal3 and a reactive sialic acid derivatized with the radioisotope.

In another exemplary embodiment, the invention provides methods for modifying Reopro, which is mutated to contain an N glycosylation site. Figures 52A to 52L contain such examples. In Figure 52B, Reopro expressed in a mammalian system is first treated with sialidase to trim back the sialic acid residues, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 52C, Reopro expressed in insect cells is modified by addition of PEGylated N-acetylglucosamine using an appropriate donor and GnT-I and/or II. In Figure 52D, Reopro expressed in yeast is first treated with Endo-H to trim back the glycosyl

groups. Subsequently, the protein is PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 52F, Reopro expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues and then PEGylated with ST3Gal1 using a donor of PEGylated sialic acid. In Figure 52G, Reopro expressed in insect cells is modified by PEGylation using a galactosyltransferase and a donor of PEG-galactose. In Figure 52H, Reopro expressed in bacterial first has N-acetylgalactosamine added using N-acetylgalactosamine transferase and a suitable donor. The protein is then PEGylated using a sialyltransferase and a donor of PEG-sialic acid. In Figure 52J, Reopro expressed in bacteria is modified in a different scheme: it is PEGylated via the action of N-acetylgalactosamine transferase, using a donor of PEGylated N-acetylgalactosamine. In Figure 52K, bacterially expressed Reopro is modified in yet another method: first, the polypeptide is contacted with N-acetylgalactosamine transferase and a donor of N-acetylgalactosamine that is derivatized with a reactive sialic acid via a linker, so that the polypeptide is attached to the reactive sialic acid via the linker and N-acetylgalactosamine. The polypeptide is then contacted with ST3Gal3 and asialo-transferrin and thus becomes connected with transferrin via the sialic acid residue. Then, the polypeptide is capped with sialic acid residues using a proper donor and ST3Gal3. Figure 52L offers an additional scheme of modifying bacterially expressed Reopro. The polypeptide is first exposed to NHS-CO-linker-SA-CMP and becomes connected with the reactive sialic acid through the linker. The polypeptide is then contacted with ST3Gal3 and asialo-transferrin and thus becomes connected with transferrin via the sialic acid residue. Then, the polypeptide is capped with sialic acid residues using a proper donor and ST3Gal3.

In another exemplary embodiment, the invention provides methods for producing Rituxan™ conjugates. Figures 53A to 53G presents some examples. In Figure 53B, Rituxan™ expressed in various mammalian systems is first galactosylated using a proper galactose donor and a galactosyltransferase. The peptide is then functionalized with a sialic acid derivatized with a toxin moiety, using a sialic acid donor and ST3Gal3. In Figure 53C, Rituxan™ expressed in mammalian cells or fungal cells is galactosylated using a galactosyltransferase and a galactose donor, which provides the peptide galactose containing a drug moiety. Figure 53D provides another example of remodeling Rituxan™ expressed in a fungal system. The polypeptide's glycosyl groups are first trimmed back using Endo-H.

Galactose is then added using a galactosyltransferase and a galactose donor. Subsequently, a radioisotope is conjugated to the molecule through a radioisotope-complexed sialic acid donor and a sialyltransferase, ST3Gal3. In Figure 53F, Rituxan™ is expressed in a mammalian system and first galactosylated using a galactosyltransferase and a proper galactose donor; sialic acid with a PEG moiety is then attached to the molecule using ST3Gal3 and a PEGylated sialic acid donor. As shown in Figure 53G, Rituxan™ expressed in fungi, yeast, or mammalian cells can also be modified in the following process: first, the polypeptide is treated with  $\alpha$ - and  $\beta$ -mannosidases to remove terminal mannosyl residues; GlcNAc is then attached to the molecule using GnT-I, II and a GlcNAc donor, radioisotope is then attached by way of galactosylation using a galactosyltransferase and a donor of galactose that is coupled to a chelating moiety capable of binding a radioisotope.

In another exemplary embodiment, the invention provides methods for modifying anti-thrombin III (AT III). Figures 54A to 54O present some examples. In Figure 54B, anti-thrombin III expressed in various mammalian systems is remodeled by the addition of one or more terminal sialic acid-PEG moieties. The AT III molecule is first contacted with sialidase to remove terminal sialic acid moieties. Then, the molecule is contacted with a sialyltransferase and an appropriate sialic acid donor that has been derivatized with a PEG moiety. In Figure 54C, AT III expressed in various mammalian systems is remodeled by the addition of sialic acid-PEG moieties. The AT III molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is then contacted with a ST3Gal3 and an appropriate sialic acid donor that has been derivatized with a PEG moiety at 1.2 mol eq. The molecule is then contacted with a ST3Gal3 and an appropriate sialic acid donor to cap remaining terminal galactose moieties. In Figure 54D, AT III is expressed in NSO murine myeloma cells is remodeled to have complex glycan molecules with terminal sialic acid-PEG moieties. The AT III molecule is contacted with sialidase and  $\alpha$ -galactosidase to remove terminal sialic acid and galactose moieties. The molecule is then contacted with galactosyltransferase and an appropriated galactose donor. The molecule is then contacted with a ST3Gal3 and an appropriate sialic acid donor that has been derivatized with a PEG moiety. In Figure 54E, AT III expressed in various mammalian systems is remodeled to have nearly complete terminal sialic acid-PEG moieties. The AT III molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is then contacted with a

ST3Gal3 and an appropriate sialic acid donor that has been derivatized with a PEG moiety at 16 mol eq. The molecule is then contacted with ST3Gal3 and an appropriate sialic acid donor to cap remaining terminal galactose moieties. In Figure 54F, AT III expressed in various mammalian systems is remodeled by the addition of one or more terminal sialic acid PEG moieties. The AT III molecule is contacted with ST3Gal3 and an appropriate sialic acid donor that has been derivatized with a levulinate moiety. The molecule is then contacted with hydrazine-PEG. In Figure 54G, AT III expressed in various mammalian systems is remodeled by the addition of one or more terminal poly- $\alpha$ 2,8-linked sialic acid moieties. The AT III molecule is contacted with poly- $\alpha$ 2,8-sialyltransferase and an appropriate sialic acid donor. In Figure 54I, AT III expressed in insect, yeast or fungi cells is remodeled by the addition of branching N- N-acetylglucosamine -PEG moieties. The AT III molecule is contacted with GnT-I and an appropriate N-acetylglucosamine donor that has been derivatized with PEG. In Figure 54J, AT III expressed in yeast is remodeled by removing high mannose glycan structures and the addition of terminal sialic acid-PEG moieties. The AT III molecule is contacted with endoglycanase to trim back glycosyl groups. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor. The molecule is then contacted with ST3Gal3 and an appropriate sialic acid donor that has been derivatized with a PEG moiety. In Figure 54K, AT III expressed in various mammalian systems is remodeled by the addition of glycoconjugated transferrin. The AT III molecule is contacted with ST3Gal3 and an appropriate sialic acid donor that has been derivatized with a linker-galactose donor moiety. The molecule is then contacted with galactosyltransferase and endoglycanase-treated transferrin. In Figure 54M, AT III expressed in yeast is remodeled by the removal of mannose glycan structures and the addition of terminal galactose-PEG moieties. The molecule is contacted with endoglycanase to trim back glycosyl groups. The molecule is further contacted with galactosyltransferase and an appropriate galactose donor that has been derivatized with a PEG moiety. In Figure 54N, AT III expressed in plant cells is remodeled by converting the glycan structures into mammalian-type complex glycans and then adding one or more terminal galactose-PEG moieties. The AT III molecule is contacted with xylosidase to remove xylose residues. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor that has been derivatized with a PEG moiety. In Figure 54O, AT III expressed in various mammalian systems is remodeled



by the addition of one or more terminal sialic acid-PEG moieties to terminal galactose moieties. The AT III molecule is contacted with ST3Gal3 and an appropriate sialic acid PEG donor that has been derivatized with PEG.

In another exemplary embodiment, the invention provides methods for modifying the  $\alpha$  and  $\beta$  subunits of human Chorionic Gonadotropin (hCG). Figures 55A to 55J present some examples. In Figure 55B, hCG expressed in various mammalian and insect systems is remodeled by the addition of terminal sialic acid-PEG moieties. The hCG molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is then contacted with ST3Gal3 and an appropriate sialic acid donor molecule that has been derivatized with a PEG moiety. In Figure 55C, hCG expressed in insect cell, yeast or fungi systems is remodeled by building out the N-linked glycans and the addition of terminal sialic acid-PEG moieties. The hCG molecule is contacted with GnT-I and GnT-II, and an appropriated N-acetylglucosamine donor. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor. The molecule is further contacted with ST3Gal3 and an appropriate sialic acid donor that has been derivatized with a PEG moiety. In Figure 55D, hCG expressed in various mammalian and insect systems is remodeled by the addition of one or more terminal sialic acid-PEG moieties on O-linked glycan structures. The hCG molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is then contacted with ST3Gal3 and an appropriate sialic acid donor to cap the glycan structures with sialic acid moieties. The molecule is then contacted with ST3Gal1 and an appropriate sialic acid donor that has been derivatized with PEG. In Figure 55E, hCG expressed in various mammalian and insect systems is remodeled by the addition of sialic acid-PEG moieties to N-linked glycan structures. The hCG molecule is contacted with ST3Gal3 and an appropriate sialic acid donor that has been derivatized with PEG. In Figure 55F, hCG expressed in insect cells, yeast or fungi, is remodeled by the addition of terminal N-acetylglucosamine-PEG molecules. The hCG molecule is contacted with GnT-I and GnT-II, and an appropriate N-acetylglucosamine donor that has been derivatized with PEG. In Figure 55G, hCG expressed in insect cells, yeast or fungi, is remodeled by the addition of not more than one N-acetylglucosamine-PEG moiety per N-linked glycan structure. The hCG molecule is contacted with GnT-I and an appropriate N-acetylglucosamine donor that has been derivatized with a PEG moiety. In Figure 55H, hCG

expressed in various mammalian systems is remodeled by the addition of one or more terminal sialic acid-PEG moiety to O-linked glycan structures. The hCG molecule is contacted with ST3Gal3 and an appropriate sialic acid donor that has been derivatized with PEG. In Figure 55I, hCG expressed in various mammalian systems is remodeled by the addition of terminal sialic acid-PEG moieties. The hCG molecule is contacted with  $\alpha$ 2,8-SA and an appropriate sialic acid donor that has been derivatized with a PEG moiety. In Figure 55J, hCG expressed in various mammalian systems is remodeled by the addition of terminal sialic acid moieties. The hCG molecule is contacted with poly-alpha2,8-ST and an appropriate sialic acid donor that has been derivatized with a PEG moiety.

In another exemplary embodiment, the invention provides methods for modifying alpha-galactosidase A (Fabrazyme™). Figures 56A to 56J present some examples. In Figure 56B, alpha-galactosidase A expressed in and secreted from various mammalian and insect systems is remodeled by the addition of one or more terminal galactose-PEG-transferrin moieties. The alpha-galactosidase A molecule is contacted with Endo-H to trim back glycosyl groups. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor that has been derivatized with PEG and transferrin. In Figure 56C, alpha-galactosidase A expressed in and secreted from various mammal and insect cell systems is remodeled by the addition of one or more terminal sialic acid-linker-mannose-6-phosphate moieties. The alpha-galactosidase A molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is further contacted with ST3Gal3 and an appropriate sialic acid donor that has been conjugated via a linker to mannose-6-phosphate. In Figure 56D, alpha-galactosidase A expressed in NSO murine myeloma cells is remodeled by the addition of terminal sialic acid-linker-mannose-6-phosphate moieties. The alpha-galactosidase A molecule is contacted with sialidase and  $\alpha$ -galactosidase to remove terminal sialic acid and galactose moieties. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor. The molecule is then contacted with sialyltransferase and an appropriate sialic acid donor that has been conjugated via a linker to mannose-6-phosphate. In Figure 56E, alpha-galactosidase A expressed in and secreted from various mammalian and insect cell systems is remodeled by the addition of one or more terminal sialic acid-PEG moieties. The alpha-galactosidase A molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is then contacted with sialyltransferase

and an appropriate sialic acid donor that has been derivatized with a PEG moiety. In Figure 56F, alpha-galactosidase A expressed in mammalian, insect, yeast or fungi systems, is remodeled by the addition of one or more terminal mannose-linker-ApoE moieties. The alpha-galactosidase A molecule is contacted with mannosyltransferase and an appropriate mannose donor that has been conjugated via a linker to ApoE. In Figure 56G, alpha-galactosidase A expressed in mammalian, insect, yeast or fungal systems is remodeled by the addition of galactose-linker-alpha2-macroglobulin moieties. The alpha-galactosidase A molecule is contacted with Endo-H to trim back glycosyl groups. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor that has been conjugated via a linker to alpha2-macroglobulin. In Figure 56H, alpha-galactosidase A expressed in insect, yeast and fungal systems, is remodeled by the addition of one or more N-acetylglucosamine-PEG-mannose-6-phosphate moieties. The alpha-galactosidase molecule is contacted with GnT-I and an appropriate N-acetyl-glucosamine donor that has been derivatized with PEG and mannose-6-phosphate. In Figure 56I, alpha-galactosidase A expressed in insect, yeast or fungal systems, is remodeled by the addition of one or more terminal galactose-PEG-transferrin moieties. The alpha-galactosidase A molecule is contacted with GnT-I and an appropriate N-acetyl-glucosamine donor. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor that has been derivatized with PEG and transferrin. In Figure 56J, alpha-galactosidase A expressed in insect, yeast or fungi systems is remodeled by the addition of one or more terminal sialic acid-PEG-melanotransferrin moieties. The alpha-galactosidase A molecule is contacted with GnT-I and GnT-II and an appropriate N-acetyl-glucosamine donor. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor. The molecule is then contacted with sialyltransferase and an appropriate sialic acid donor that has been derivatized with PEG and melanotransferrin.

In another exemplary embodiment, the invention provides methods for modifying alpha-iduronidase (Aldurazyme™). Figures 57A to 57J present some examples. In Figure 57B, alpha-iduronidase expressed in and secreted from various mammalian and insect systems is remodeled by the addition of one or more terminal galactose-PEG-transferrin moieties. The alpha-iduronidase molecule is contacted with Endo-H to trim back glycosyl groups. The molecule is then contacted with galactosyltransferase and an appropriate

galactose donor that has been derivatized with PEG and transferrin. In Figure 57C, alpha-iduronidase expressed in and secreted from various mammal and insect cell systems is remodeled by the addition of terminal sialic acid-linker-mannose-6-phosphate moieties. The alpha-iduronidase molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is then contacted with ST3Gal3 and an appropriate sialic acid donor that has been conjugated via a linker to mannose-6-phosphate. In Figure 57D, alpha-iduronidase expressed in NSO murine myeloma cells is remodeled by the addition of one or more terminal sialic acid-linker-mannose-6-phosphate moieties. The alpha-iduronidase molecule is contacted with sialidase and  $\alpha$ -galactosidase to remove terminal sialic acid and galactose moieties. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor. The molecule is further contacted with sialyltransferase and an appropriate sialic acid donor that has been conjugated via a linker to mannose-6-phosphate. In Figure 57E, alpha-iduronidase expressed in and secreted from various mammalian and insect cell systems is remodeled by the addition of one or more terminal sialic acid-PEG moieties. The alpha-iduronidase molecule is contacted with sialidase to remove terminal sialic acid moieties. The molecule is further contacted with sialyltransferase and an appropriate sialic acid donor that has been derivatized with a PEG moiety. In Figure 57F, alpha-iduronidase expressed in mammalian, insect, yeast or fungi systems is remodeled by the addition of one or more terminal mannose-linker-ApoE moieties. The alpha-iduronidase molecule is contacted with mannosyltransferase and an appropriate mannose donor that has been conjugated via a linker to ApoE. In Figure 57G, alpha-iduronidase expressed in mammalian, insect, yeast or fungal systems is remodeled by the addition of one or more galactose-linker-alpha2-macroglobulin moieties. The alpha-iduronidase molecule is contacted with Endo-H to trim back glycosyl groups. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor that has been conjugated via a linker to alpha2-macroglobulin. In Figure 57H, alpha-iduronidase expressed in insect, yeast and fungal systems, is remodeled by the addition of one or more N-acetylglucosamine-PEG-mannose-6-phosphate moieties. The alpha-galactosidase molecule is contacted with GnT-I and an appropriate N-acetyl-glucosamine donor that has been derivatized with PEG and mannose-6-phosphate. In Figure 57I, alpha-iduronidase expressed in insect, yeast or fungal systems, is remodeled by the addition of one or more terminal galactose-PEG-transferrin

moieties. The alpha-iduronidase molecule is contacted with GnT-I and an appropriate N-acetyl-glucosamine donor. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor that has been derivatized with PEG and transferrin. In Figure 57J, alpha-iduronidase expressed in insect, yeast or fungi systems, is remodeled by the addition of one or more terminal sialic acid-PEG-melanotransferrin moieties. The alpha-iduronidase molecule is contacted with GnT-I and GnT-II and an appropriate N-acetyl-glucosamine donor. The molecule is then contacted with galactosyltransferase and an appropriate galactose donor. The molecule is further contacted with sialyltransferase and an appropriate sialic acid donor that has been derivatized with PEG and melanotransferrin.

#### A. Creation or elimination of N-linked glycosylation sites

The present invention contemplates the use of peptides in which the site of the glycan chain(s) on the peptide have been altered from that of the native peptide. Typically, N-linked glycan chains are linked to the primary peptide structure at asparagine residues where the asparagine residue is within an amino acid sequence that is recognized by a membrane-bound glycosyltransferase in the endoplasmic reticulum (ER). Typically, the recognition site on the primary peptide structure is the sequence asparagine-X-serine/threonine where X can be any amino acid except proline and aspartic acid. While this recognition site is typical, the invention further encompasses peptides that have N-linked glycan chains at other recognition sites where the N-linked chains are added using natural or recombinant glycosyltransferases.

Since the recognition site for N-linked glycosylation of a peptide is known, it is within the skill of persons in the art to create mutated primary peptide sequences wherein a native N-linked glycosylation recognition site is removed, or alternatively or in addition, one or more additional N-glycosylation recognition sites are created. Most simply, an asparagine residue can be removed from the primary sequence of the peptide thereby removing the attachment site for a glycan, thus removing one glycan from the mature peptide. For example, a native recognition site with the sequence of asparagine-serine-serine can be genetically engineered to have the sequence leucine-serine-serine, thus eliminating a N-linked glycosylation site at this position.

Further, an N-linked glycosylation site can be removed by altering the residues in the recognition site so that even though the asparagine residue is present, one or more of the

additional recognition residues are absent. For example, a native sequence of asparagine-serine-serine can be mutated to asparagine-serine-lysine, thus eliminating an N-glycosylation site at that position. In the case of N-linked glycosylation sites comprising residues other than the typical recognition sites described above, the skilled artisan can determine the sequence and residues required for recognition by the appropriate glycosyltransferase, and then mutate at least one residue so the appropriate glycosyltransferase no longer recognizes that site. In other words, it is well within the skill of the artisan to manipulate the primary sequence of a peptide such that glycosylation sites are either created or are removed, or both, thereby generating a peptide having an altered glycosylation pattern. The invention should therefore not be construed to be limited to any primary peptide sequence provided herein as the sole sequence for glycan remodeling, but rather should be construed to include any and all peptide sequences suitable for glycan remodeling.

To create a mutant peptide, the nucleic acid sequence encoding the primary sequence of the peptide is altered so that native codons encoding native amino acid residues are mutated to generate a codon encoding another amino acid residue. Techniques for altering nucleic acid sequence are common in the art and are described for example in any well-known molecular biology manual.

In addition, the nucleic acid encoding a primary peptide structure can be synthesized *in vitro*, using standard techniques. For example, a nucleic acid molecule can be synthesized in a "gene machine" using protocols such as the phosphoramidite method. If chemically-synthesized double stranded DNA is required for an application such as the synthesis of a nucleic acid or a fragment thereof, then each complementary strand is synthesized separately. The production of short nucleic acids (60 to 80 base pairs) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer nucleic acids (>300 base pairs), special strategies may be required, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length. For reviews on polynucleotide synthesis, see, for example, Glick and Pasternak (Molecular Biotechnology, Principles and Applications of Recombinant DNA,

1994, ASM Press), Itakura et al. (1984, Annu. Rev. Biochem. 53:323), and Climie et al. (1990, Proc. Nat'l Acad. Sci. USA 87:633).

Additionally, changes in the nucleic acid sequence encoding the peptide can be made by site-directed mutagenesis. As will be appreciated, this technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site-directed mutagenesis which eliminates the step of transferring the nucleic acid of interest from a plasmid to a phage.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector or melting the two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform or transfect appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement. A genetic selection scheme was devised by Kunkel et al. (1987, Kunkel et al., Methods Enzymol. 154:367-382) to enrich for clones incorporating the mutagenic oligonucleotide. Alternatively, the use of PCR<sup>TM</sup> with commercially available thermostable enzymes such as Taq polymerase may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an appropriate cloning or expression vector. The PCR<sup>TM</sup>-mediated mutagenesis procedures of Tomic et al. (1990, Nucl. Acids Res., 12:1656) and Upender et al. (1995, Biotechniques, 18:29-31) provide two examples of such protocols. A PCR<sup>TM</sup> employing a thermostable ligase in addition to a thermostable polymerase may also be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment that may then be cloned into an appropriate cloning or expression vector. The mutagenesis procedure described by Michael (1994, Biotechniques 16:410-412) provides an example of one such protocol.

Not all Asn-X-Ser/Thr sequences are N-glycosylated suggesting the context in which the motif is presented is important. In another approach, libraries of mutant peptides having novel N-linked consensus sites are created in order to identify novel N-linked sites that are glycosylated *in vivo* and are beneficial to the activity, stability or other characteristics of the peptide.

As noted previously, the consensus sequence for the addition of N-linked glycan chains in glycoproteins is Asn-X-Ser/Thr where X can be any amino acid. The nucleotide sequence encoding the amino acid two positions to the carboxyl terminal side of the Asn may be mutated to encode a Ser and/or Thr residue using standard procedures known to those of ordinary skill in the art. As stated above not all Asn-X-Ser/Thr sites are modified by the addition of glycans. Therefore, each recombinant mutated glycoprotein must be expressed in a fungal, yeast or animal or mammalian expression system and analyzed for the addition of an N-linked glycan chain. The techniques for the characterization of glycosylation sites are well known to one skilled in the art. Further, the biological function of the mutated recombinant glycoprotein can be determined using assays standard for the particular protein being examined. Thus, it becomes a simple matter to manipulate the primary sequence of a peptide and identify novel glycosylation sites contained therein, and further determine the effect of the novel site on the biological activity of the peptide.

In an alternative embodiment, the nucleotide sequence encoding the amino acid two positions to the amino terminal side of Ser/Thr residues may be mutated to encode an Asn using standard procedures known to those of ordinary skill in the art. The procedures to determine whether a novel glycosylation site has been created and the effect of this site on the biological activity of the peptide are described above.

#### B. Creation or elimination of O-linked glycosylation sites

The addition of an O-linked glycosylation site to a peptide is conveniently accomplished by altering the primary amino acid sequence of the peptide such that it contains one or more additional O-linked glycosylation sites compared with the beginning primary amino acid sequence of the peptide. The addition of an O-linked glycosylation site to the peptide may also be accomplished by incorporation of one or more amino acid species into the peptide which comprises an -OH group, preferably serine or threonine residues, within the sequence of the peptide, such that the OH group is accessible and available for O-linked



glycosylation. Similar to the discussion of alteration of N-linked glycosylation sites in a peptide, the primary amino acid sequence of the peptide is preferably altered at the nucleotide level. Specific nucleotides in the DNA sequence encoding the peptide may be altered such that a desired amino acid is encoded by the sequence. Mutation(s) in DNA are preferably made using methods known in the art, such as the techniques of phosphoramidite method DNA synthesis and site-directed mutagenesis described above.

Alternatively, the nucleotide sequence encoding a putative site for O-linked glycan addition can be added to the DNA molecule in one or several copies to either 5' or the 3' end of the molecule. The altered DNA sequence is then expressed in any one of a fungal, yeast, or animal or mammalian expression system and analyzed for the addition of the sequence to the peptide and whether or not this sequence is a functional O-linked glycosylation site. Briefly, a synthetic peptide acceptor sequence is introduced at either the 5' or 3' end of the nucleotide molecule. In principle, the addition of this type of sequence is less disruptive to the resulting glycoprotein when expressed in a suitable expression system. The altered DNA is then expressed in CHO cells or other suitable expression system and the proteins expressed thereby are examined for the presence of an O-linked glycosylation site. In addition, the presence or absence of glycan chains can be determined.

In yet another approach, advantageous sites for new O-linked sites may be found in a peptide by creating libraries of the peptide containing various new O-linked sites. For example, the consensus amino acid sequence for N-acetylgalactosamine addition by an N-acetylgalactosaminyltransferase depends on the specific transferase used. The amino acid sequence of a peptide may be scanned to identify contiguous groups of amino acids that can be mutated to generate potential sites for addition of O-linked glycan chains. These mutations can be generated using standard procedures known to those of ordinary skill in the art as described previously. In order to determine if any discovered glycosylation site is actually glycosylated, each recombinant mutated peptide is then expressed in a suitable expression system and is subsequently analyzed for the addition of the site and/or the presence of an O-linked glycan chain.

### C. Chemical synthesis of peptides

While the primary structure of peptides useful in the invention can be generated most efficiently in a cell-based expression system, it is within the scope of the present invention

that the peptides may be generated synthetically. Chemical synthesis of peptides is well known in the art and include, without limitation, stepwise solid phase synthesis, and fragment condensation either in solution or on solid phase. A classic stepwise solid phase synthesis of involves covalently linking an amino acid corresponding to the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending the peptide chain toward the amino end by stepwise coupling of activated amino acid derivatives having activated carboxyl groups. After completion of the assembly of the fully protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is cleaved by suitable chemistry and the protecting groups are removed to yield the product peptide. See, R. Merrifield, Solid Phase Peptide Synthesis: The Synthesis of a Tetrapeptide, J. Am. Chem. Soc., 85:2149-2154 (1963). The longer the peptide chain, the more challenging it is to obtain high-purity well-defined products. Due to the production of complex mixtures, the stepwise solid phase synthesis approach has size limitations. In general, well-defined peptides of 100 contiguous amino acid residues or more are not routinely prepared via stepwise solid phase synthesis.

The segment condensation method involves preparation of several peptide segments by the solid phase stepwise method, followed by cleavage from the solid phase and purification of these maximally protected segments. The protected segments are condensed one-by-one to the first segment, which is bound to the solid phase.

The peptides useful in the present invention may be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. These synthesis methods are well-known to those of skill in the art (see, for example, Merrifield, J. Am. Chem. Soc. 85:2149 (1963), Stewart et al., "Solid Phase Peptide Synthesis" (2nd Edition), (Pierce Chemical Co. 1984), Bayer and Rapp, Chem. Pept. Prot. 3:3 (1986), Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach (IRL Press 1989), Fields and Colowick, "Solid-Phase Peptide Synthesis," Methods in Enzymology Volume 289 (Academic Press 1997), and Lloyd-Williams et al., Chemical Approaches to the Synthesis of Peptides and Peptides (CRC Press, Inc. 1997)). Variations in total chemical synthesis strategies, such as "native chemical ligation" and "expressed peptide ligation" are also standard (see, for example, Dawson et al., Science 266:776 (1994), Hackeng et al., Proc. Nat'l Acad. Sci. USA 94:7845 (1997), Dawson, Methods Enzymol. 287: 34 (1997), Muir et al, Proc. Nat'l Acad. Sci. USA 95:6705 (1998), and Severinov and Muir, J. Biol. Chem.

273:16205 (1998)). Also useful are the solid phase peptide synthesis methods developed by Gryphon Sciences, South San Francisco, CA. See, U.S. Patent Nos. 6,326,468, 6,217,873, 6,174,530, and 6,001,364, all of which are incorporated in their entirety by reference herein.

5        D. Post-translational modifications

It will be appreciated to one of ordinary skill in the art that peptides may undergo post-translational modification besides the addition of N-linked and/or O-linked glycans thereto. It is contemplated that peptides having post-translational modifications other than glycosylation can be used as peptides in the invention, as long as the desired biological  
10    activity or function of the peptide is maintained or improved. Such post-translational modifications may be natural modifications usually carried out *in vivo*, or engineered modifications of the peptide carried out *in vitro*. Contemplated known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a  
15    nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cysteine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing,  
20    phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to peptides such as arginylation, and ubiquitination. Enzymes that may be used to carry out many of these modifications are well known in the art, and available commercially from companies such as Boehringer Mannheim (Indianapolis, IN) and Sigma Chemical Company (St. Louis, MO), among others.

25        Such modifications are well known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as Peptides--Structure and Molecular Properties, 2nd Ed., T. E. Creighton, W. H. Freeman and  
30    Company, New York (1993). Many detailed reviews are available on this subject, such as by Wold, F., Post-translational Covalent Modification of Peptides, B. C. Johnson, Ed., Academic

Press, New York 1-12 (1983); Seifter et al. (Meth. Enzymol. 182: 626-646 (1990)) and Rattan et al. (Ann. N.Y. Acad. Sci. 663:48-62 (1992)).

Covalent modifications of a peptide may also be introduced into the molecule *in vitro* by reacting targeted amino-acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal amino-acid residues. Most commonly derivatized residues are cysteinyl, histidyl, lysinyl, arginyl, tyrosyl, glutaminyl, asparaginyl and amino terminal residues. Hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl and threonyl residues, methylation of the alpha-amino groups of lysine, histidine, and histidine side chains, acetylation of the N-terminal amine and amidation of the C-terminal carboxylic groups. Such derivatized moieties may improve the solubility, absorption, biological half life and the like. The moieties may also eliminate or attenuate any undesirable side effect of the peptide and the like.

In addition, derivatization with bifunctional agents is useful for cross-linking the peptide to water insoluble support matrices or to other macromolecular carriers. Commonly used cross-linking agents include glutaraldehyde, N-hydroxysuccinimide esters, homobifunctional imidoesters, 1,1-bis(-diazoloacetyl)-2-phenylethane, and bifunctional maleimides. Derivatizing agents such as methyl-3-[9p-azidophenyl]dithiopropioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287 and 3,691,016 may be employed for peptide immobilization.

#### E. Fusion peptides/peptides

Peptides useful in the present invention may comprise fusion peptides. Fusion peptides are particularly advantageous where biological and/or functional characteristics of two peptides are desired to be combined in one peptide molecule. Such fusion peptides can present combinations of biological activity and function that are not found in nature to create novel and useful molecules of therapeutic and industrial applications. Biological activities of interest include, but are not limited to, enzymatic activity, receptor and/or ligand activity, immunogenic motifs, and structural domains.

Such fusion peptides are well known in the art, and the methods of creation will be well-known to those in the art. For example, a human  $\alpha$ -interferon—human albumin fusion peptide has been made wherein the resulting peptide has the therapeutic benefits of  $\alpha$ -interferon combined with the long circulating life of albumin, thereby creating a therapeutic composition that allows reduced dosing frequency and potentially reduced side effects in patients. See, Albuferon™ from Human Genome Sciences, Inc. and U.S. Patent No. 5,766,883. Other fusion peptides include antibody molecules that are described elsewhere herein.

10      F. Generation of smaller “biologically active” molecules

The peptides used in the invention may be variants of native peptides, wherein a fragment of the native peptide is used in place of the full length native peptide. In addition, pre-pro-, and pre-peptides are contemplated. Variant peptides may be smaller in size than the native peptide, and may comprise one or more domains of a larger peptide. Selection of specific peptide domains can be advantageous when the biological activity of certain domains in the peptide is desired, but the biological activity of other domains in the peptide is not desired. Also included are truncations of the peptide and internal deletions which may enhance the desired therapeutic effect of the peptide. Any such forms of a peptide is contemplated to be useful in the present invention provided that the desired biological activity of the peptide is preserved.

Shorter versions of peptides may have unique advantages not found in the native peptide. In the case of human albumin, it has been found that a truncated form comprising as little as 63% of the native albumin peptide is advantageous as a plasma volume expander. The truncated albumin peptide is considered to be better than the native peptide for this therapeutic purpose because an individual peptide dose of only one-half to two-thirds that of natural-human serum albumin, or recombinant human serum albumin is required for the equivalent colloid osmotic effect. See U.S. Patent No. 5,380,712, the entirety of which is incorporated by reference herein.

Smaller “biologically active” peptides have also been found to have enhanced therapeutic activity as compared to the native peptide. The therapeutic potential of IL-2 is limited by various side effects dominated by the vascular leak syndrome. A shorter

chemically synthesized version of the peptide consisting of residues 1-30 corresponding to the entire  $\alpha$ -helix was found to fold properly and contain the natural IL-2 biological activity with out the attending side effects.

#### G. Generation of novel peptides

5           The peptide of the invention may be derived from a primary sequence of a native peptide, or may be engineered using any of the many means known to those of skill in the art. Such engineered peptides can be designed and/or selected because of enhanced or novel properties as compared with the native peptide. For example, peptides may be engineered to have increased enzyme reaction rates, increased or decreased binding affinity  
10 to a substrate or ligand, increased or decreased binding affinity to a receptor, altered specificity for a substrate, ligand, receptor or other binding partner, increased or decreased stability *in vitro* and/or *in vivo*, or increased or decreased immunogenicity in an animal.

#### H. Mutations

##### 1. Rational design mutation

15           The peptides useful in the methods of the invention may be mutated to enhance a desired biological activity or function, to diminish an undesirable property of the peptide, and/or to add novel activities or functions to the peptide. "Rational peptide design" may be used to generate such altered peptides. Once the amino acid sequence and structure of the peptide is known and a desired mutation planned, the mutations can be made most  
20 conveniently to the corresponding nucleic acid codon which encodes the amino acid residue that is desired to be mutated. One of skill in the art can easily determine how the nucleic acid sequence should be altered based on the universal genetic code, and knowledge of codon preferences in the expression system of choice. A mutation in a codon may be made to change the amino acid residue that will be polymerized into the peptide during translation.  
25 Alternatively, a codon may be mutated so that the corresponding encoded amino acid residue is the same, but the codon choice is better suited to the desired peptide expression system. For example, cys-residues may be replaced with other amino acids to remove disulfide bonds from the mature peptide, catalytic domains may be mutated to alter biological activity, and in general, isoforms of the peptide can be engineered. Such mutations can be point mutations,  
30 deletions, insertions and truncations, among others.

Techniques to mutate specific amino acids in a peptide are well known in the art. The technique of site-directed mutagenesis, discussed above, is well suited for the directed mutation of codons. The oligonucleotide-mediated mutagenesis method is also discussed in detail in Sambrook et al. (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, starting at page 15.51). Systematic deletions, insertions and truncations can be made using linker insertion mutagenesis, digestion with nuclease Bal31, and linker-scanning mutagenesis, among other methods well known to those in the art (Sambrook et al., 2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York).

Rational peptide design has been successfully used to increase the stability of enzymes with respect to thermoinactivation and oxidation. For example, the stability of an enzyme was improved by removal of asparagine residues in  $\alpha$ -amylase (Declerck et al., 2000, *J. Mol. Biol.* 301:1041-1057), the introduction of more rigid structural elements such as proline into  $\alpha$ -amylase (Igarashi et al., 1999, *Biosci. Biotechnol. Biochem.* 63:1535-1540) and D-xylose isomerase (Zhu et al., 1999, *Peptide Eng.* 12:635-638). Further, the introduction of additional hydrophobic contacts stabilized 3-isopropylmalate dehydrogenase (Akanuma et al., 1999, *Eur. J. Biochem.* 260:499-504) and formate dehydrogenase obtained from *Pseudomonas* sp. (Rojkova et al., 1999, *FEBS Lett.* 445:183-188). The mechanisms behind the stabilizing effect of these mutations is generally applicable to many peptides. These and similar mutations are contemplated to be useful with respect to the peptides remodeled in the methods of the present invention.

## 2. Random mutagenesis techniques

Novel peptides useful in the methods of the invention may be generated using techniques that introduce random mutations in the coding sequence of the nucleic acid. The nucleic acid is then expressed in a desired expression system, and the resulting peptide is assessed for properties of interest. Techniques to introduce random mutations into DNA sequences are well known in the art, and include PCR mutagenesis, saturation mutagenesis, and degenerate oligonucleotide approaches. See Sambrook and Russell (2001, *Molecular Cloning, A Laboratory Approach*, Cold Spring Harbor Press, Cold Spring Harbor, NY) and Ausubel et al. (2002, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY).

In PCR mutagenesis, reduced Taq polymerase fidelity is used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, Technique 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations into a DNA sequence. The DNA region to be mutagenized is amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using an altered dGTP/dATP ratio and by adding  $Mn^{2+}$  to the PCR reaction. The pool of amplified DNA fragments are inserted into appropriate cloning vectors to provide random mutant libraries.

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, Science 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complementary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments, both neutral substitutions as well as those that alter function, are obtained. The distribution of point mutations is not biased toward conserved sequence elements.

A library of nucleic acid homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of a degenerate oligonucleotide sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes may then be ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp. 273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other peptides (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Pat. Nos. 5,223,409, 5,198,346, and 5,096,815).

a. Directed evolution



Peptides useful in the methods of the invention may also be generated using "directed evolution" techniques. In contrast to site directed mutagenesis techniques where knowledge of the structure of the peptide is required, there now exist strategies to generate libraries of mutations from which to obtain peptides with improved properties without knowledge of the structural features of the peptide. These strategies are generally known as "directed evolution" technologies and are different from traditional random mutagenesis procedures in that they involve subjecting the nucleic acid sequence encoding the peptide of interest to recursive rounds of mutation, screening and amplification.

In some "directed evolution" techniques, the diversity in the nucleic acids obtained is generated by mutation methods that randomly create point mutations in the nucleic acid sequence. The point mutation techniques include, but are not limited to, "error-prone PCR™" (Caldwell and Joyce, 1994; PCR Methods Appl. 2: 28-33; and Ke and Madison, 1997, Nucleic Acids Res. 25: 3371-3372), repeated oligonucleotide-directed mutagenesis (Reidhaar-Olson et al., 1991, Methods Enzymol. 208:564-586), and any of the aforementioned methods of random mutagenesis.

Another method of creating diversity upon which directed evolution can act is the use of mutator genes. The nucleic acid of interest is cultured in a mutator cell strain the genome of which typically encodes defective DNA repair genes (U.S. Patent No. 6,365,410; Selifonova et al., 2001, Appl. Environ. Microbiol. 67:3645-3649; Long-McGie et al., 2000, Biotech. Bioeng. 68:121-125; see, Genencor International Inc, Palo Alto CA).

Achieving diversity using directed evolution techniques may also be accomplished using saturation mutagenesis along with degenerate primers (Gene Site Saturation Mutagenesis™, Diversa Corp., San Diego, CA). In this type of saturation mutagenesis, degenerate primers designed to cover the length of the nucleic acid sequence to be diversified are used to prime the polymerase in PCR reactions. In this manner, each codon of a coding sequence for an amino acid may be mutated to encode each of the remaining common nineteen amino acids. This technique may also be used to introduce mutations, deletions and insertions to specific regions of a nucleic acid coding sequence while leaving the rest of the nucleic acid molecule untouched. Procedures for the gene saturation technique are well known in the art, and can be found in U.S. Patent 6,171,820.

#### b. DNA shuffling

Novel peptides useful in the methods of the invention may also be generated using the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling techniques are may be employed to modulate the activities of peptides useful in the invention and may be used to generate peptides having altered activity. See, generally, U.S. Pat. Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Stemmer et al. (1994, Nature 370(6488):389-391); Crameri et al. (1998, Nature 391 (6664):288-291); Zhang et al. (1997, Proc. Natl. Acad. Sci. USA 94(9):4504-4509); Stemmer et al. (1994, Proc. Natl. Acad. Sci. USA 91(22):10747-10751), Patten et al. (1997, Curr. Opinion Biotechnol. 8:724-33); Harayama, (1998, Trends Biotechnol. 16(2):76-82); Hansson, et al., (1999, J. Mol. Biol. 287:265-76); and Lorenzo and Blasco (1998, Biotechniques 24(2):308-13) (each of these patents are hereby incorporated by reference in its entirety).

DNA shuffling involves the assembly of two or more DNA segments by homologous or site-specific recombination to generate variation in the polynucleotide sequence. DNA shuffling has been used to generate novel variations of human immunodeficiency virus type 1 proteins (Pekrun et al., 2002, J. Virol. 76(6):2924-35), triazine hydrolases (Raillard et al. 2001, Chem Biol 8(9):891-898), murine leukemia virus (MLV) proteins (Powell et al. 2000, Nat Biotechnol 18(12):1279-1282), and indoleglycerol phosphate synthase (Merz et al. 2000, Biochemistry 39(5):880-889).

The technique of DNA shuffling was developed to generate biomolecular diversity by mimicking natural recombination by allowing *in vitro* homologous recombination of DNA (Stemmler, 1994, Nature 370: 389-391; and Stemmler, 1994, PNAS 91: 10747-10751). Generally, in this method a population of related genes is fragmented and subjected to recursive cycles of denaturation, rehybridization, followed by the extension of the 5' overhangs by Taq polymerase. With each cycle, the length of the fragments increases, and DNA recombination occurs when fragments originating from different genes hybridize to each other. The initial fragmentation of the DNA is usually accomplished by nuclease digestion, typically using DNase (see Stemmler references, above), but may also be accomplished by interrupted PCR synthesis (U.S. Patent 5,965,408, incorporated herein by reference in its entirety; see, Diversa Corp., San Diego, CA). DNA shuffling methods have advantages over random point mutation methods, in that direct recombination of beneficial

mutations generated by each round of shuffling is achieved and there is therefore a self selection for improved phenotypes of peptides.

The techniques of DNA shuffling are well known to those in art. Detailed explanations of such technology is found in Stemmler, 1994, Nature 370: 389-391 and Stemmler, 1994, PNAS 91: 10747-10751. The DNA shuffling technique is also described in U.S. Patents 6,180,406, 6,165,793, 6,132,970, 6,117,679, 6,096,548, 5,837,458, 5,834,252, 5,830,721, 5,811,238, and 5,605,793 (all of which are incorporated by reference herein in their entirety).

The art also provides even more recent modifications of the basic technique of DNA shuffling. In one example, exon shuffling, exons or combinations of exons that encode specific domains of peptides are amplified using chimeric oligonucleotides. The amplified molecules are then recombined by self-priming PCR assembly (Kolkman and Stemmler, 2001, Nat. Biotech. 19:423-428). In another example, using the technique of random chimeragenesis on transient templates (RACHITT) library construction, single stranded parental DNA fragments are annealed onto a full-length single-stranded template (Coco et al., 2001, Nat. Biotechnol. 19:354-359). In yet another example, staggered extension process (StEP), thermocycling with very abbreviated annealing/extension cycles is employed to repeatedly interrupt DNA polymerization from flanking primers (Zhao et al., 1998, Nat. Biotechnol. 16: 258-261). In the technique known as CLERY, *in vitro* family shuffling is combined with *in vivo* homologous recombination in yeast (Abecassis et al., 2000, Nucleic Acids Res. 28:E88; ). To maximize intergenic recombination, single stranded DNA from complementary strands of each of the nucleic acids are digested with DNase and annealed (Kikuchi et al., 2000, Gene 243:133-137). The blunt ends of two truncated nucleic acids of variable lengths that are linked by a cleavable sequence are then ligated to generate gene fusion without homologous recombination (Sieber et al., 2001, Nat Biotechnol. 19:456-460; Lutz et al., 2001, Nucleic Acids Res. 29:E16; Ostermeier et al., 1999, Nat. Biotechnol. 17:1205-1209; Lutz and Benkovic, 2000, Curr. Opin. Biotechnol. 11:319-324). Recombination between nucleic acids with little sequence homology in common has also been enhanced using exonuclease-mediated blunt-ending of DNA fragments and ligating the fragments together to recombine them (U.S. Patent No. 6,361,974, incorporated herein by reference in its entirety). The invention contemplates the use of each and every variation

described above as a means of enhancing the biological properties of any of the peptides and/or enzymes useful in the methods of the invention.

In addition to published protocols detailing directed evolution and gene shuffling techniques, commercial services are now available that will undertake the gene shuffling and selection procedures on peptides of choice. Maxygen (Redwood City, CA) offers commercial services to generate custom DNA shuffled libraries. In addition, this company will perform customized directed evolution procedures including gene shuffling and selection on a peptide family of choice.

Optigenix, Inc. (Newark, DE) offers the related service of plasmid shuffling. Optigenix uses families of genes to obtain mutants therein having new properties. The nucleic acid of interest is cloned into a plasmid in an *Aspergillus* expression system. The DNA of the related family is then introduced into the expression system and recombination in conserved regions of the family occurs in the host. Resulting mutant DNAs are then expressed and the peptide produced therefrom are screened for the presence of desired properties and the absence of undesired properties.

#### c. Screening procedures

Following each recursive round of "evolution," the desired peptides expressed by mutated genes are screened for characteristics of interest. The "candidate" genes are then amplified and pooled for the next round of DNA shuffling. The screening procedure used is highly dependant on the peptide that is being "evolved" and the characteristic of interest. Characteristics such as peptide stability, biological activity, antigenicity, among others can be selected using procedures that are well known in the art. Individual assays for the biological activity of preferred peptides useful in the methods of the invention are described elsewhere herein.

#### d. Combinations of techniques

It will be appreciated by the skilled artisan that the above techniques of mutation and selection can be combined with each other and with additional procedures to generate the best possible peptide molecule useful in the methods of the invention. Thus, the invention is not limited to any one method for the generation of peptides, and should be construed to encompass any and all of the methodology described herein. For example, a procedure for introducing point mutations into a nucleic acid sequence may be performed initially, followed

by recursive rounds of DNA shuffling, selection and amplification. The initial introduction of point mutations may be used to introduce diversity into a gene population where it is lacking, and the following round of DNA shuffling and screening will select and recombine advantageous point mutations.

5

### III. Glycosidases and Glycotransferases

#### A. Glycosidases

Glycosidases are glycosyltransferases that use water as an acceptor molecule, and as such, are typically glycoside-hydrolytic enzymes. Glycosidases can be used for the formation of glycosidic bonds *in vitro* by controlling the thermodynamics or kinetics of the reaction mixture. Even with modified reaction conditions, though, glycosidase reactions can be difficult to work with, and glycosidases tend to give low synthetic yields as a result of the reversible transglycosylase reaction and the competing hydrolytic reaction.

A glycosidase can function by retaining the stereochemistry at the bond being broken during hydrolysis or by inverting the stereochemistry at the bond being broken during hydrolysis, classifying the glycosidase as either a "retaining" glycosidase or an "inverting" glycosidase, respectively. Retaining glycosidases have two critical carboxylic acid moieties present in the active site, with one carboxylate acting as an acid/base catalyst and the other as a nucleophile, whereas with the inverting glycosidases, one carboxylic acid functions as an acid and the other functions as a base.

Methods to determine the activity and linkage specificity of any glycosidase are well known in the art, including a simplified HPLC protocol (Jacob and Scudder, 1994, Methods in Enzymol. 230: 280-300). A general discussion of glycosidases and glycosidase treatment is found in Glycobiology, A Practical Approach, (1993, Fukuda and Kobata eds., Oxford University Press Inc., New York).

Glycosidases useful in the invention include, but are not limited to, sialidase, galactosidase, endoglycanase, mannosidase (i.e.,  $\alpha$  and  $\beta$ , ManI, ManII and ManIII,) xylosidase, fucosidase, *Agrobacterium* sp.  $\beta$ -glucosidase, *Cellulomonas fimi* mannosidase 2A, *Humicola insolens* glycosidase, *Sulfolobus solfataricus* glycosidase and *Bacillus licheniformis* glycosidase.

The choice of fucosidases for use in the invention depends on the linkage of the fucose to other molecules. The specificities of many  $\alpha$ -fucosidases useful in the methods of

the invention are well known to those in the art, and many varieties of fucosidase are also commercially available (Glyko, Novato, CA; PROzyme, San Leandro, CA; Calbiochem-Novabiochem Corp., San Diego, CA; among others).  $\alpha$ -Fucosidases of interest include, but are not limited to,  $\alpha$ -fucosidases from *Turbo cornutus*, *Charonia lampas*, *Bacillus fulminans*,  
5 *Aspergillus niger*, *Clostridium perfringens*, Bovine kidney (Glyko), chicken liver (Tyagarajan et al., 1996, Glycobiology 6:83-93) and  $\alpha$ -fucosidase II from *Xanthomonas manihotis* (Glyko, PROzyme). Chicken liver fucosidase is particularly useful for removal of core fucose from N-linked glycans.

#### 10 B. Glycosyltransferases

Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase and a lipid-linked oligosaccharide donor Dol-PP-NAG<sub>2</sub>Glc<sub>3</sub>Man<sub>9</sub> in an en block transfer followed  
15 by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art.

The glycosyltransferase to be used in the present invention may be any as long as it can utilize the modified sugar as a sugar donor. Examples of such enzymes include Leloir  
20 pathway glycosyltransferases, such as galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, fucosyltransferase, sialyltransferase, mannosyltransferase, xylosyltransferase, glucurononyltransferase and the like.

For enzymatic saccharide syntheses that involve glycosyltransferase reactions,  
25 glycosyltransferases can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. See, e.g., Taniguchi et al., 2002, Handbook of glycosyltransferases and related genes, Springer, Tokyo.

Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino acid sequences can be deduced are also found in  
30 various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

Glycosyltransferases that can be employed in the methods of the invention include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those  
5 obtained from eukaryotes, as well as from prokaryotes.

DNA encoding glycosyltransferases may be obtained by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures.

10 Screening of mRNA or genomic DNA may be carried out using oligonucleotide probes generated from the glycosyltransferases nucleic acid sequence. Probes may be labeled with a detectable label, such as, but not limited to, a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays. In the alternative, glycosyltransferases nucleic acid sequences may be  
15 obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the glycosyltransferases nucleic acid sequence. See, U.S. Pat. No. 4,683,195 to Mullis et al. and U.S. Pat. No. 4,683,202 to Mullis.

A glycosyltransferases enzyme may be synthesized in a host cell transformed with a vector containing DNA encoding the glycosyltransferases enzyme. A vector is a replicable  
20 DNA construct. Vectors are used either to amplify DNA encoding the glycosyltransferases enzyme and/or to express DNA which encodes the glycosyltransferases enzyme. An expression vector is a replicable DNA construct in which a DNA sequence encoding the glycosyltransferases enzyme is operably linked to suitable control sequences capable of effecting the expression of the glycosyltransferases enzyme in a suitable host. The need for  
25 such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the  
30 ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

### 1. Fucosyltransferases

In some embodiments, a glycosyltransferase used in the method of the invention is a fucosyltransferase. Fucosyltransferases are known to those of skill in the art. Exemplary fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer from non-nucleotide sugars to an acceptor are also of use in the present invention.

In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Gal $\beta$ (1 $\rightarrow$ 3,4)GlcNAc $\beta$ - group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the Gal $\beta$ (1 $\rightarrow$ 3,4)GlcNAc $\beta$ 1- $\alpha$ (1 $\rightarrow$ 3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (see, Palcic, et al., Carbohydrate Res. 190: 1-11 (1989); Prieels, et al., J. Biol. Chem. 256: 10456-10463 (1981); and Nunez, et al., Can. J. Chem. 59: 2086-2095 (1981)) and the Gal $\beta$ (1 $\rightarrow$ 4)GlcNAc $\beta$ - $\alpha$ fucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 2.4.1.65), a sialyl  $\alpha$ (2 $\rightarrow$ 3)Gal $\beta$ ((1 $\rightarrow$ 3)GlcNAc $\beta$  fucosyltransferase, has also been characterized. A recombinant form of the Gal $\beta$ (1 $\rightarrow$ 3,4) GlcNAc $\beta$ - $\alpha$ (1 $\rightarrow$ 3,4)fucosyltransferase has also been characterized (see, Dumas, et al., Bioorg. Med. Letters 1: 425-428 (1991) and Kukowska-Latallo, et al., Genes and Development 4: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example,  $\alpha$ 1,2 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, et al., Eur. J. Biochem. 191: 169-176 (1990) or U.S. Patent No. 5,374,655.

### 2. Galactosyltransferases

In another group of embodiments, the glycosyltransferase is a galactosyltransferase.

Exemplary galactosyltransferases include  $\alpha$ (1,3) galactosyltransferases (E.C. No. 2.4.1.151, see, e.g., Dabkowski et al., Transplant Proc. 25:2921 (1993) and Yamamoto et al. Nature 345: 229-233 (1990), bovine (GenBank j04989, Joziase et al., J. Biol. Chem. 264: 14290-14297 (1989)), murine (GenBank m26925; Larsen et al., Proc. Nat'l. Acad. Sci. USA 86: 8227-8231 (1989)), porcine (GenBank L36152; Strahan et al., Immunogenetics 41: 101-105 (1995)).

Another suitable  $\alpha$ 1,3 galactosyltransferase is that which is involved in synthesis of the blood



group B antigen (EC 2.4.1.37, Yamamoto *et al.*, *J. Biol. Chem.* **265**: 1146-1151 (1990) (human)).

Also suitable for use in the methods of the invention are  $\beta$ (1,4) galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro *et al.*, *Eur. J. Biochem.* **183**: 211-217 (1989)), human (Masri *et al.*, *Biochem. Biophys. Res. Commun.* **157**: 657-663 (1988)), murine (Nakazawa *et al.*, *J. Biochem.* **104**: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl *et al.*, *J. Neurosci. Res.* **38**: 234-242 (1994)). Other suitable galactosyltransferases include, for example,  $\alpha$ 1,2 galactosyltransferases (from e.g., *Schizosaccharomyces pombe*, Chapell *et al.*, *Mol. Biol. Cell* **5**: 519-528 (1994)). For further suitable galactosyltransferases, see Taniguchi *et al.* (2002, *Handbook of Glycosyltransferases and Related Genes*, Springer, Tokyo), Guo *et al.* (2001, *Glycobiology*, **11**(10):813-820), and Breton *et al.* (1998, *J Biochem.* **123**:1000-1009).

The production of proteins such as the enzyme GalNAc T<sub>I-XIV</sub> from cloned genes by genetic engineering is well known. See, e.g., U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by *in vitro* glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

### 3. Sialyltransferases

Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (e.g., a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et*

al., *Glycobiology* 6: v-xiv (1996)). An exemplary  $\alpha(2,3)$ sialyltransferase referred to as  $\alpha(2,3)$ sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Gal $\beta$ 1 $\rightarrow$ 3Glc disaccharide or glycoside. See, Van den Eijnden et al., *J. Biol. Chem.* 256: 3159 (1981), Weinstein et al., *J. Biol. Chem.* 257: 13845 (1982) and Wen et al., *J. Biol.*

5 Chem. 267: 21011 (1992). Another exemplary  $\alpha 2,3$ -sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. see, Rearick et al., *J. Biol. Chem.* 254: 4444 (1979) and Gillespie et al., *J. Biol. Chem.* 267: 21004 (1992).

Further exemplary enzymes include Gal- $\beta$ -1,4-GlcNAc  $\alpha$ -2,6 sialyltransferase (See, Kurosawa et al. *Eur. J. Biochem.* 219: 375-381 (1994)).

10 Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Gal $\beta$ 1,4GlcNAc-, Gal $\beta$ 1,3GlcNAc-, or Gal $\beta$ 1,3GalNAc-, the most common penultimate sequences underlying the terminal sialic acid on fully sialylated carbohydrate structures (see, Table 8).  $\alpha 2,8$ -Sialyltransferases capable of transferring sialic acid to  $\alpha 2,3$ Gal $\beta$ 1,4GlcNAc are also useful in the methods of the  
15 invention.

**Table 8.** Sialyltransferases which use the Gal $\beta$ 1,4GlcNAc sequence as an acceptor substrate

Sialyltransferase	Source	Sequence(s) formed	Ref.
ST6Gal I	Mammalian	NeuAca2,6Gal $\beta$ 1,4GlcNAc-	1
ST3Gal III	Mammalian	NeuAca2,3Gal $\beta$ 1,4GlcNAc- NeuAca2,3Gal $\beta$ 1,3GlcNAc-	1
ST3Gal IV	Mammalian	NeuAca2,3Gal $\beta$ 1,4GlcNAc- NeuAca2,3Gal $\beta$ 1,3GlcNAc-	1
ST6Gal II	Mammalian	NeuAca2,6Gal $\beta$ 1,4GlcNAc-	
ST6Gal II	<i>Photobacterium</i>	NeuAca2,6Gal $\beta$ 1,4GlcNAc-	2
ST3Gal V	<i>N. meningitides</i> <i>N. gonorrhoeae</i>	NeuAca2,3Gal $\beta$ 1,4GlcNAc-	3

- 20 1) Goochee et al., *Bio/Technology* 9: 1347-1355 (1991)  
2) Yamamoto et al., *J. Biochem.* 120: 104-110 (1996)  
3) Gilbert et al., *J. Biol. Chem.* 271: 28271-28276 (1996)

An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as  $\alpha(2,3)$ sialyltransferase (EC 2.4.99.6). This enzyme catalyzes the transfer of sialic acid to the Gal of a Gal $\beta$ 1,3GlcNAc or Gal $\beta$ 1,4GlcNAc glycoside (see, e.g., Wen et al., J. Biol. Chem. 267: 21011 (1992); Van den Eijnden et al., J. Biol. Chem. 256: 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an  $\alpha$ -linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein et al., J. Biol. Chem. 257: 13845 (1982)); the human cDNA (Sasaki et al. (1993) J. Biol. Chem. 268: 22782-22787; Kitagawa & Paulson (1994) J. Biol. Chem. 269: 1394-1401) and genomic (Kitagawa et al. (1996) J. Biol. Chem. 271: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression. In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

An example of a sialyltransferase that is useful in the claimed methods is CST-I from *Campylobacter* (see, for example, U.S. Pat. No. 6,503,744, 6,096,529, and 6,210,933 and WO99/49051, and published U.S. Pat. Application 2002/2,042,369). This enzyme catalyzes the transfer of sialic acid to the Gal of a Gal $\beta$ 1,4Glc or Gal $\beta$ 1,3GalNAc. Other exemplary sialyltransferases of use in the present invention include those isolated from *Campylobacter jejuni*, including the  $\alpha(2,3)$  sialyltransferase. See, e.g., WO99/49051.

Other sialyltransferases, including those listed in Table 8, are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. As a simple test to find out the utility of these other enzymes, various amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo- $\alpha_1$  AGP (at 1-10 mg/ml) to compare the ability of the sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo- $\alpha_1$  AGP for this evaluation. Sialyltransferases with the ability to sialylate N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation (as illustrated for ST3Gal III in this disclosure).

#### 4. Other glycosyltransferases

One of skill in the art will understand that other glycosyltransferases can be substituted into similar transferase cycles as have been described in detail for the sialyltransferase. In particular, the glycosyltransferase can also be, for instance, glucosyltransferases, e.g., Alg8 (Stagljev et al., Proc. Natl. Acad. Sci. USA 91: 5977 (1994)) or Alg5 (Heesen et al., Eur. J. Biochem. 224: 71 (1994)).

N-acetylgalactosaminyltransferases are also of use in practicing the present invention. Suitable N-acetylgalactosaminyltransferases include, but are not limited to,  $\alpha(1,3)$  N-acetylgalactosaminyltransferase,  $\beta(1,4)$  N-acetylgalactosaminyltransferases (Nagata et al., J. Biol. Chem. 267: 12082-12089 (1992) and Smith et al., J. Biol. Chem. 269: 15162 (1994)) and peptide N-acetylgalactosaminyltransferase (Homa et al., J. Biol. Chem. 268: 12609 (1993)). Suitable N-acetylglucosaminyltransferases include GnT-I (2.4.1.101, Hull et al., BBRC 176: 608 (1991)), GnT-II, GnT-III (Ihara et al., J. Biochem. 113: 692 (1993)), GnT-IV, GnT-V (Shoreibah et al., J. Biol. Chem. 268: 15381 (1993)) and GnT-VI, O-linked N-acetylglucosaminyltransferase (Bierhuizen et al., Proc. Natl. Acad. Sci. USA 89: 9326 (1992)), N-acetylglucosamine-1-phosphate transferase (Rajput et al., Biochem J. 285: 985 (1992)), and hyaluronan synthase.

Mannosyltransferases are of use to transfer modified mannose moieties. Suitable mannosyltransferases include  $\alpha(1,2)$  mannosyltransferase,  $\alpha(1,3)$  mannosyltransferase,  $\alpha(1,6)$  mannosyltransferase,  $\beta(1,4)$  mannosyltransferase, Dol-P-Man synthase, OCh1, and Pmt1 (see, Kornfeld et al., Annu. Rev. Biochem. 54: 631-664 (1985)).

Xylosyltransferases are also useful in the present invention. See, for example, Rodgers, et al., Biochem. J., 288:817-822 (1992); and Elbain, et al., U.S. Patent No., 6,168,937.

Other suitable glycosyltransferase cycles are described in Ichikawa et al., JACS 114: 9283 (1992), Wong et al., J. Org. Chem. 57: 4343 (1992), and Ichikawa et al. in CARBOHYDRATES AND CARBOHYDRATE POLYMERS. Yaltami, ed. (ATL Press, 1993).

Prokaryotic glycosyltransferases are also useful in practicing the invention. Such glycosyltransferases include enzymes involved in synthesis of lipooligosaccharides (LOS), which are produced by many gram negative bacteria. The LOS typically have terminal glycan sequences that mimic glycoconjugates found on the surface of human epithelial cells

or in host secretions (Preston et al., Critical Reviews in Microbiology 23(3): 139-180 (1996)). Such enzymes include, but are not limited to, the proteins of the *rfa* operons of species such as *E. coli* and *Salmonella typhimurium*, which include a  $\beta$ 1,6 galactosyltransferase and a  $\beta$ 1,3 galactosyltransferase (see, e.g., EMBL Accession Nos. M80599 and M86935 (*E. coli*);

5 EMBL Accession No. S56361 (*S. typhimurium*)), a glucosyltransferase (Swiss-Prot Accession No. P25740 (*E. coli*), an  $\beta$ 1,2-glucosyltransferase (*rfaJ*)(Swiss-Prot Accession No. P27129 (*E. coli*) and Swiss-Prot Accession No. P19817 (*S. typhimurium*)), and an  $\beta$ 1,2-N-acetylglucosaminyltransferase (*rfaK*)(EMBL Accession No. U00039 (*E. coli*). Other glycosyltransferases for which amino acid sequences are known include those that are

10 encoded by operons such as *rfaB*, which have been characterized in organisms such as *Klebsiella pneumoniae*, *E. coli*, *Salmonella typhimurium*, *Salmonella enterica*, *Yersinia enterocolitica*, *Mycobacterium leprosum*, and the *rhI* operon of *Pseudomonas aeruginosa*.

Also suitable for use in the present invention are glycosyltransferases that are involved in producing structures containing lacto-N-neotetraose, D-galactosyl- $\beta$ -1,4-N-

15 acetyl-D-glucosaminyl- $\beta$ -1,3-D-galactosyl- $\beta$ -1,4-D-glucose, and the P<sup>k</sup> blood group trisaccharide sequence, D-galactosyl- $\alpha$ -1,4-D-galactosyl- $\beta$ -1,4-D-glucose, which have been identified in the LOS of the mucosal pathogens *Neisseria gonorrhoeae* and *N. meningitidis* (Scholten et al., J. Med. Microbiol. 41: 236-243 (1994)). The genes from *N. meningitidis* and *N. gonorrhoeae* that encode the glycosyltransferases involved in the biosynthesis of these

20 structures have been identified from *N. meningitidis* immunotypes L3 and L1 (Jennings et al., Mol. Microbiol. 18: 729-740 (1995)) and the *N. gonorrhoeae* mutant F62 (Gotshlich, J. Exp. Med. 180: 2181-2190 (1994)). In *N. meningitidis*, a locus consisting of three genes, *lgtA*, *lgtB* and *lgtE*, encodes the glycosyltransferase enzymes required for addition of the last three

25 of the sugars in the lacto-N-neotetraose chain (Wakarchuk et al., J. Biol. Chem. 271: 19166-73 (1996)). Recently the enzymatic activity of the *lgtB* and *lgtA* gene product was demonstrated, providing the first direct evidence for their proposed glycosyltransferase function (Wakarchuk et al., J. Biol. Chem. 271(45): 28271-276 (1996)). In *N. gonorrhoeae*, there are two additional genes, *lgtD* which adds  $\beta$ -D-GalNAc to the 3 position of the terminal galactose of the lacto-N-neotetraose structure and *lgtC* which adds a terminal  $\alpha$ -D-Gal to the

30 lactose element of a truncated LOS, thus creating the P<sup>k</sup> blood group antigen structure

(Gotshlich (1994), *supra.*). In *N. meningitidis*, a separate immunotype L1 also expresses the P<sup>k</sup> blood group antigen and has been shown to carry an *lgtC* gene (Jennings *et al.*, (1995), *supra.*). *Neisseria* glycosyltransferases and associated genes are also described in USPN 5,545,553 (Gotschlich). Genes for  $\alpha$ 1,2-fucosyltransferase and  $\alpha$ 1,3-fucosyltransferase from  
5 *Helicobacter pylori* has also been characterized (Martin *et al.*, J. Biol. Chem. 272: 21349-21356 (1997)). Also of use in the present invention are the glycosyltransferases of *Campylobacter jejuni* (see, Taniguchi *et al.*, 2002, Handbook of glycosyltransferases and related genes, Springer, Tokyo).

#### B. Sulfotransferases

10 The invention also provides methods for producing peptides that include sulfated molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta *et al.*, J. Biol. Chem. 270: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-  
15 acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon *et al.*, Genomics 26: 239-241 (1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana *et al.*, J. Biol. Chem. 269: 2270-2276 (1994) and Eriksson *et al.*, J. Biol. Chem. 269: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

#### 20 C. Cell-Bound Glycosyltransferases

In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are  
25 considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular  
30 recognition (Roth, 1990, Molecular Approaches to Supracellular Phenomena,).

Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen et al., Proc. Natl. Acad. Sci. USA 86: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:β-D-galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

Francisco et al., Proc. Natl. Acad. Sci. USA 89: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to prokaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

#### D. Fusion Enzymes

In other exemplary embodiments, the methods of the invention utilize fusion peptides that have more than one enzymatic activity that is involved in synthesis of a desired glycopeptide conjugate. The fusion peptides can be composed of, for example, a catalytically active domain of a glycosyltransferase that is joined to a catalytically active domain of an accessory enzyme. The accessory enzyme catalytic domain can, for example, catalyze a step in the formation of a nucleotide sugar that is a donor for the glycosyltransferase, or catalyze a reaction involved in a glycosyltransferase cycle. For example, a polynucleotide that encodes a glycosyltransferase can be joined, in-frame, to a polynucleotide that encodes an enzyme involved in nucleotide sugar synthesis. The resulting fusion peptide can then catalyze not only the synthesis of the nucleotide sugar, but also the transfer of the sugar moiety to the acceptor molecule. The fusion peptide can be two or more cycle enzymes linked into one expressible nucleotide sequence. In other embodiments the fusion peptide includes the catalytically active domains of two or more glycosyltransferases. See, for example, U.S. Patent No. 5,641,668. The modified glycopeptides of the present invention can be readily

designed and manufactured utilizing various suitable fusion peptides (*see*, for example, PCT Patent Application PCT/CA98/01180, which was published as WO 99/31224 on June 24, 1999.)

#### E. Immobilized Enzymes

5 In addition to cell-bound enzymes, the present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the methods of the invention. The PEG-linker-enzyme conjugate is optionally attached to solid support. The use of solid supported enzymes in the methods of  
10 the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of skill in the art.

#### F. Mutagenesis of Glycosyltransferases

15 The novel forms of the glycosyltransferases, sialyltransferases, sulfotransferases, and any other enzymes used in the method of the invention can be created using any of the methods described previously, as well as others well known to those in the art. Of particular interest are transferases with altered acceptor specificity and/or donor specificity. Also of interest are enzymes with higher conversion rates and higher stability among others.

20 The techniques of rational design mutagenesis can be used when the sequence of the peptide is known. Since the sequences as well as many of the tertiary structures of the transferases and glucosidases used in the invention are known, these enzymes are ideal for rational design of mutants. For example, the catalytic site of the enzyme can be mutated to alter the donor and/or acceptor specificity of the enzyme.

25 The extensive tertiary structural data on the glycosyltransferases and glycosidase hydrolases also make these enzyme idea for mutations involving domain exchanges. Glycosyltransferases and glycosidase hydrolases are modular enzymes (*see*, Bourne and Henrissat, 2001, *Current Opinion in Structural Biology* 11:593-600). Glycosyltransferases are divided into two families bases on their structure: GT-A and GT-B. The  
30 glycosyltransferases of the GT-A family comprise two dissimilar domains, one involved in nucleotide binding and the other in acceptor binding. Thus, one could conveniently fuse the



DNA sequence encoding the domain from one gene in frame with a domain from a second gene to create a new gene that encodes a protein with a new acceptor/donor specificity. Such exchanges of domains could additionally include the carbohydrate modules and other accessory domains.

5       The techniques of random mutation and/or directed evolution, as described above, may also be used to create novel forms of the glycosyltransferases and glycosidases used in the invention.

#### IV. *In vitro* and *in vivo* expression systems

##### 10       A. Cells for the production of glycopeptides

The action of glycosyltransferases is key to the glycosylation of peptides, thus, the difference in the expression of a set of glycosyltransferases in any given cell type affects the pattern of glycosylation on any given peptide produced in that cell. For a review of host cell dependent glycosylation of peptides, see Kabata and Takasaki, "Structure and Biosynthesis of  
15 Cell Surface Carbohydrates," in Cell Surface Carbohydrates and Cell Development, 1991, pp. 1-24, Eds. Minoru Fukuda, CRC Press, Boca Raton, FL.

According to the present disclosure, the type of cell in which the peptide is produced is relevant only with respect to the degree of remodeling required to generate a peptide having desired glycosylation. For example, the number and sequence of enzymatic digestion  
20 reactions and the number and sequence of enzymatic synthetic reactions that are required *in vitro* to generate a peptide having desired glycosylation will vary depending on the structure of the glycan on the peptide produced by a particular cell type. While the invention should in no way be construed to be limited to the production of peptides from any one particular cell type including any cell type disclosed herein, a discussion of several cell systems is now  
25 presented which establishes the power of the present invention and its independence of the cell type in which the peptides are generated.

In general, and to express a peptide from a nucleic acid encoding it, the nucleic acid must be incorporated into an expression cassette, comprising a promoter element, a terminator element, and the coding sequence of the peptide operably linked between the two.  
30 The expression cassette is then operably linked into a vector. Toward this end, adapters or linkers may be employed to join the nucleotide fragments or other manipulations may be

involved to provide for convenient restriction sites, removal of superfluous nucleotides, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved. A shuttle vector has the genetic elements necessary for replication in a cell. Some vectors may be replicated only in prokaryotes, or may be replicated in both prokaryotes and eukaryotes. Such a plasmid expression vector will be maintained in one or more replication systems, preferably two replication systems, that allow for stable maintenance within a yeast host cell for expression purposes, and within a prokaryotic host for cloning purposes. Many vectors with diverse characteristics are now available commercially. Vectors are usually plasmids or phages, but may also be cosmids or mini-chromosomes. Conveniently, many commercially available vectors will have the promoter and terminator of the expression cassette already present, and a multi-linker site where the coding sequence for the peptide of interest can be inserted. The shuttle vector containing the expression cassette is then transformed in *E. coli* where it is replicated during cell division to generate a preparation of vector that is sufficient to transform the host cells of the chosen expression system. The above methodology is well known to those in the art, and protocols by which to accomplish can be found Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

The vector, once purified from the cells in which it is amplified, is then transformed into the cells of the expression system. The protocol for transformation depended on the kind of the cell and the nature of the vector. Transformants are grown in an appropriate nutrient medium, and, where appropriate, maintained under selective pressure to insure retention of endogenous DNA. Where expression is inducible, growth can be permitted of the yeast host to yield a high density of cells, and then expression is induced. The secreted, mature heterologous peptide can be harvested by any conventional means, and purified by chromatography, electrophoresis, dialysis, solvent-solvent extraction, and the like.

The techniques of molecular cloning are well-known in the art. Further, techniques for the procedures of molecular cloning can be found in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Glover et al., (1985, DNA Cloning: A Practical Approach, Volumes I and II); Gait et al., (1985, Oligonucleotide Synthesis); Hames and Higgins (1985, Nucleic Acid

Hybridization ); Hames and Higgins (1984, Transcription And Translation); Freshney et al., (1986, Animal Cell Culture); Perbal, (1986, Immobilized Cells And Enzymes, IRL Press); Perbal,(1984, A Practical Guide To Molecular Cloning); Ausubel et al. (2002, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.).

5        B. Fungi and yeast

Peptides produced in yeast are glycosylated and the glycan structures present thereon are primarily high mannose structures. In the case of N-glycans, the glycan structures produced in yeast may contain as many as nine or more mannose residues which may or may not contain additional sugars added thereto. An example of the type of glycan on peptides produced by yeast cells is shown in Figure 4, left side. Irrespective of the number of mannose residues and the type and complexity of additional sugars added thereto, N-glycans as components of peptides produced in yeast cells comprise a trimannosyl core structure as shown in Figure 4. When the glycan structure on a peptide produced by a yeast cell is a high mannose structure, it is a simple matter for the ordinary skilled artisan to remove, *in vitro* using available mannosidase enzymes, all of the mannose residues from the molecule except for those that comprise the trimannosyl core of the glycan, thereby generating a peptide having an elemental trimannosyl core structure attached thereto. Now, using the techniques available in the art and armed with the present disclosure, it is a simple matter to enzymatically add, *in vitro*, additional sugar moieties to the elemental trimannosyl core structure to generate a peptide having a desired glycan structure attached thereto. Similarly, when the peptide produced by the yeast cell comprises a high mannose structure in addition to other complex sugars attached thereto, it is a simple matter to enzymatically cleave off all of the additional sugars, including extra mannose residues, to arrive at the elemental trimannosyl core structure. Once the elemental trimannosyl core structure is produced, generation of a peptide having desired glycosylation is possible following the directions provided herein.

By "yeast" is intended ascosporogenous yeasts (Endomycetales), basidiosporogenous yeasts, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into two families, Spermophthoraceae and Saccharomycetaceae. The later is comprised of four subfamilies, *Schizosaccharomycoideae* (e.g., genus *Schizosaccharomyces*), *Nadsonioideae*, *Lipomycoideae*, and *Saccharomycoideae* (e.g., genera

*Pichia*, *Kluyveromyces*, and *Saccharomyces*). The basidiosporogenous yeasts include the genera *Leucosporidium*, *Rhodospiridium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeast belonging to the Fungi Imperfecti are divided into two families, *Sporobolomycetaceae* (e.g., genera *Sporobolomyces*, *Bullera*) and *Cryptococcaceae* (e.g., genus *Candida*). Of particular interest to the present invention are species within the genera *Saccharomyces*, *Pichia*, *Aspergillus*, *Trichoderma*, *Kluyveromyces*, especially *K. lactis* and *K. drosophilum*, *Candida*, *Hansenula*, *Schizosaccharomyces*, *Yarrowia*, and *Chrysosporium*. Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Skinner et al., eds. 1980) *Biology and Activities of Yeast* (Soc. App. Bacteriol. Symp. Series No. 9).

In addition to the foregoing, those of ordinary skill in the art are presumably familiar with the biology of yeast and the manipulation of yeast genetics. See, for example, Bacila et al., eds. (1978, *Biochemistry and Genetics of Yeast*, Academic Press, New York); and Rose and Harrison. (1987, *The Yeasts* (2<sup>nd</sup> ed.) Academic Press, London). Methods of introducing exogenous DNA into yeast hosts are well known in the art. There are a wide variety of methods for transformation of yeast. Spheroplast transformation is taught by Hinnen et al (1978, *Proc. Natl. Acad. Sci. USA* 75:1919-1933); Beggs, (1978, *Nature* 275(5676):104-109); and Stinchcomb et al., (EPO Publication No. 45,573; herein incorporated by reference), Electroporation is taught by Becker and Gaurante, (1991, *Methods Enzymol.* 194:182-187), Lithium acetate is taught by Gietz et al. (2002, *Methods Enzymol.* 350:87-96) and Mount et al. (1996, *Methods Mol Biol.* 53:139-145). For a review of transformation systems of non-*Saccharomyces* yeasts, see Wang et al. (*Crit Rev Biotechnol.* 2001;21(3):177-218). For general procedures on yeast genetic engineering, see Barr et al., (1989, *Yeast genetic engineering*, Butterworths, Boston).

In addition to wild-type yeast and fungal cells, there are also strains of yeast and fungi that have been mutated and/or selected to enhance the level of expression of the exogenous gene, and the purity, the post-translational processing of the resulting peptide, and the recovery and purity of the mature peptide. Expression of an exogenous peptide may also be direct to the cell secretory pathway, as illustrated by the expression of insulin (see (Kjeldsen, 2000, *Appl. Microbiol. Biotechnol.* 54:277-286, and references cited therein). In general, to cause the exogenous peptide to be secreted from the yeast cell, secretion signals derived from

yeast genes may be used, such as those of the genes of the killer toxin (Stark and Boyd, 1986, EMBO J. 5:1995-2002) or of the alpha pheromone (Kurjan and Herskowitz, 1982, Cell 30:933; Brake et al., 1988, Yeast 4:S436).

Regarding the filamentous fungi in general, methods for genetic manipulation can be found in Kinghorn and Turner (1992, Applied Molecular Genetics of Filamentous Fungi, Blackie Academic and Professional, New York). Guidance on appropriate vectors can be found in Martinelli and Kinghorn (1994, Aspergillus : 50 years, Elsevier, Amsterdam).

### 1. Saccharomyces

In *Saccharomyces*, suitable yeast vectors for use producing a peptide include YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76: 1035-1039, 1978), YEpl3 (Broach et al., Gene 8: 121-133, 1979), POT vectors (Kawasaki et al, U.S. Pat. No. 4,931,373, which is incorporated by reference herein), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof. Preferred promoters for use in yeast include promoters for yeast glycolytic gene expression (Hitzeman et al., J. Biol. Chem. 255: 12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1: 419-434, 1982; Kawasaki, U.S. Pat. No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101: 192-201, 1983), and the ADH2-4<sup>c</sup> promoter (Russell et al., Nature 304: 652-654, 1983; Irani and Kilgore, U.S. patent application Ser. No. 07/784,653, CA 1,304,020 and EP 284 044, which are incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, *ibid.*).

Examples of such yeast-bacteria shuttle vectors include Yep24 (Botstein et al. (1979) Gene 8:17-24; pC1 (Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642-4646), and Yrp17 (Stnichomb et al. (1982) J. Mol. Biol. 158:157). Additionally, a plasmid expression vector may be a high or low copy number plasmid, the copy number generally ranging from about 1 to about 200. In the case of high copy number yeast vectors, there will generally be at least 10, preferably at least 20, and usually not exceeding about 150 copies of the vector in a single host. Depending upon the heterologous peptide selected, either a high or low copy number vector may be desirable, depending upon the effect of the vector and the recombinant

peptide on the host. See, for example, Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642-4646. DNA constructs of the present invention can also be integrated into the yeast genome by an integrating vector. Examples of such vectors are known in the art. See, for example, Botstein et al. (1979) Gene 8:17-24.

5           The selection of suitable yeast and other microorganism hosts for the practice of the present invention is within the skill of the art. Of particular interest are the *Saccharomyces* species *S. cerevisiae*, *S. carlsbergensis*, *S. diastaticus*, *S. douglasii*, *S. kluyveri*, *S. norbensis*, and *S. oviformis*. When selecting yeast host cells for expression of a desired peptide, suitable host cells may include those shown to have, inter alia, good secretion capacity, low  
10   proteolytic activity, and overall vigor. Yeast and other microorganisms are generally available from a variety of sources, including the Yeast Genetic Stock Center, Department of Biophysics and Medical Physics, University of California, Berkeley, Calif.; and the American Type Culture Collection, Manassas VA. For a review, see Strathern et al., eds. (1981, The Molecular Biology of the Yeast *Saccharomyces*, Cold Spring Harbor Laboratory, Cold  
15   Spring Harbor, N.Y.)  
Methods of introducing exogenous DNA into yeast hosts are well known in the art.

## 2. Pichia

The use of *Pichia methanolica* as a host cell for the production of recombinant peptides is disclosed in PCT Applications WO 97/17450, WO 97/17451, WO 98/02536, and  
20   WO 98/02565. DNA molecules for use in transforming *P. methanolica* are commonly prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For peptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (AUG1 or AUG2). Other useful promoters include those of the  
25   dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes, as well as those disclosed in U.S. Patent No. 5,252,726. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use  
in *Pichia methanolica* is a *P. methanolica* ADE2 gene, which encodes phosphoribosyl-5-  
30   aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows ade2 host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize

the use of methanol, host cells in which both methanol utilization genes (AUG1 and AUG2) are deleted are preferred. For production of secreted peptides, host cells deficient in vacuolar protease genes (PEP4 and PRB1) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a peptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds. For a review of the use of *Pichia pastoris* for large-scale production of antibody fragments, see Fischer et al., (1999, Biotechnol Appl Biochem. 30 (Pt 2):117-120).

### 3. Aspergillus

Methods to express peptides in *Aspergillus* spp. are well known in the art, including but not limited to those described in Carrez et al., 1990, Gene 94:147-154; Contreras, 1991, Bio/Technology 9:378-381; Yelton et al., 1984, Proc. Natl. Acad. Sci. USA 81:1470-1474; Tilburn et al., 1983, Gene 26:205-221; Kelly and. Hynes, 1985, EMBO J. 4:475-479; Ballance et al., 1983, Biochem. Biophys. Res. Comm. 112:284-289; Buxton et al., 1985, Gene 37:207-214, and U.S. Pat. No. 4,935,349, incorporated by reference herein in its entirety. Examples of promoters useful in *Aspergillus* are found in U.S. Patent No. 5,252,726. Strains of *Aspergillus* useful for peptide expression are found in U.S. Patent No. 4,935,349. Commercial production of exogenous peptides is available from Novoenzymes for *Aspergillus niger* and *Aspergillus oryzae*.

### 4. Trichoderma

*Trichoderma* has certain advantages over other species of recombinant host cells for expression of desired peptides. This organism is easy to grow in large quantities and it has the ability to glycosylate and efficiently secrete high yields of recombinant mammalian peptides into the medium, making isolation of the peptide relatively easy. In addition, the glycosylation pattern on expressed peptides is more similar to that on human peptides than peptides expressed in many other systems. However, there are still differences in the glycan structures on expressed peptides from these cells. For example, terminal sialic acid residues are important to the therapeutic function of a peptide in a mammalian system, since the presence of these moieties at the end of the glycan structure impedes peptide clearance from

the mammalian bloodstream. The mechanism behind the increased biologic half-life of sialylated molecules is believed to lie in their decreased recognition by lectins (Drickamer, 1988, J. Biol. Chem. 263:9557-9560). However, in general fungal cells do not add terminal sialic acid residues to glycans on peptides, and peptides synthesized in fungal cells are therefore asialic. According to the present invention, this deficiency can be remedied using the *in vitro* glycan remodeling methods of the invention described in detail elsewhere herein.

*Trichoderma* species useful as hosts for the production of peptides to be remodeled include *T. reesei*, such as QM6a, ALKO2442 or CBS383.78 (Centraalbureau voor Schimmelcultures, Oosterstraat 1, PO Box 273, 3740 AG Baarn, The Netherlands, or, ATCC13631 (American Type Culture Collection, Manassas VA, 10852, USA, type); *T. viride* (such as CBS189.79 (det. W. Gams); *T. longibrachiatum*, such as CBS816.68 (type); *T. pseudokoningii* (such as MUCL19358; Mycotheque de l'Universite Catholique de Louvain); *T. saturnisporum* CBS330.70 (type); *T. harzianum* CBS316.31 (det. W. Gams); *T. virgatum* (*T. pseudokoningii*) ATCC24961. Most preferably, the host is *T. reesei* and more preferably, it is *T. reesei* strains QM9414 (ATCC 26921), RUT-C-30 (ATCC 56765), and highly productive mutants such as VTT-D-79125, which is derived from QM9414 (Nevalainen, Technical Research Centre of Finland Publications 26, (1985), Espoo, Finland).

The transformation of *Trichoderma* with DNA is performed using any technique known in the art, including that taught in European patent No. EP0244234, Harkki (1989, Bio/Technology 7:596-601) and Uusitalo (1991, J. Biotech. 17:35-50). Culture of *Trichoderma* is supported by previous extensive experience in industrial scale fermentation techniques; for example, see Finkelstein, 1992, Biotechnology of Filamentous Fungi: Technology and Products, Butterworth-Heinemann, publishers, Stoneham, Mass.

#### 5. *Kluyveromyces*

Yeast belonging to the genus *Kluyveromyces* have been used as host organisms for the production of recombinant peptides. Peptides produced by this genus of yeast are, in particular, chymosin (European Patent 96 430), thaumatin (European Patent 96 910), albumin, interleukin-1 $\beta$ , TPA, TIMP (European Patent 361 991) and albumin derivatives having a therapeutic function (European Patent 413 622). Species of particular interest in the genus *Kluyveromyces* include *K. lactis*.



Methods of expressing recombinant peptides in *Kluyvermyces* spp. are well known in the art. Vectors for the expression and secretion of human recombinant peptides in *Kluyvermyces* are known in the art (Yeh, J. Cell. Biochem. Suppl. 14C:68, Abst. H402; Fleer, 1990, Yeast 6 (Special Issue):S449) as are procedures for transformation and expression of recombinant peptides (Ito et al., 1983, J. Bacteriol. 153:163-168; van den Berg, 1990, Bio/Technology 8:135-139; U.S. Patent No. 5,633,146, WO8304050A1, EP0096910, EP0241435, EP0301670, EP0361991, all of which are incorporated by reference herein in their entirety). For a review of genetic manipulation of *Kluyveromyces lactis* linear DNA plasmids by gene targeting and plasmid shuffles, see Schaffrath et al. (1999, FEMS Microbiol Lett. 178(2):201-210).

#### 6. *Chrysosporium*

The fungal genus *Chrysosporium* has recently been used to expression of foreign recombinant peptides. A description of the procedures by which one of skill in the art can use *Chrysosporium* can be used to express foreign peptides is found in WO 00/20555 (incorporated by reference herein in its entirety). Species particularly suitable for expression system include, but are not limited to, *C. botryoides*, *C. carmichaelii*, *C. crassitunicatum*, *C. europae*, *C. evolceannui*, *F. fastidium*, *C. filiforme*, *C. gerogiae*, *C. globiferum*, *C. globiferum* var. *articulatum*, *C. globiferum* var. *niveum*, *C. hirundo*, *C. hispanicum*, *C. holmii*, *C. indicum*, *C. inops*, *C. keratinophilum*, *C. kreiselii*, *C. kuzurovianum*, *C. lignorum*, *C. lobatum*, *C. lucknowense*, *C. lucknowense* Garg 27K, *C. medium*, *C. medium* var. *spissescens*, *C. mephiticum*, *C. merdarium*, *C. merdarium* var. *roseum*, *C. minor*, *C. pannicola*, *C. parvum*, *C. parvum* var. *crescens*, *C. pilosum*, *C. peodomerderium*, *C. pyriformis*, *C. queenslandicum*, *C. sigleri*, *C. sulfureum*, *C. synchronum*, *C. tropicum*, *C. undulatum*, *C. vallenarens*, *C. vespertilium*, and *C. zonatum*.

#### 7. Others

Methods for transforming *Schwanniomyces* are disclosed in European Patent 394 538. Methods for transforming *Acremonium chrysogenum* are disclosed by U.S. Pat. No. 5,162,228. Methods for transforming *Neurospora* are disclosed by U.S. Pat. No. 4,486,533. Also known is an expression system specifically for *Schizosaccharomyces pombe* (European

Patent 385 391). General methods for expressing peptides in fission yeast, *Schizosaccharomyces pombe* can be found in Giga-Hama and Kumagai (1997, Foreign gene expression in fission yeast : *Schizosaccharomyces pombe*, Springer, Berlin).

### 5        C. Mammalian systems

As discussed above, mammalian cells typically produce a heterogeneous mixture of N-glycan structures which vary with respect to the number and arrangement of additional sugars attached to the trimannosyl core. Typically, mammalian cells produce peptides having a complex glycan structure, such as that shown in Figure 3, right side. Using the methods of  
10 the present invention, a peptide produced in a mammalian cell may be remodeled *in vitro* to generate a peptide having desired glycosylation by first identifying the primary glycan structure and then determining which sugars must be removed in order to remodel the glycan structure. As discussed herein, the sugars to be removed will determine which cleavage enzymes will be used and thus, the precise steps of the remodeling process will vary  
15 depending on the primary glycan structure used as the initial substrate. A sample scheme for remodeling a glycan structure commonly produced in mammalian cells is shown in Figure 2. The N-glycan biosynthetic pathway in mammalian cells has been well characterized (reviewed in Moremen, 1994, Glycobiology 4:113-125). Many of the enzymes necessary for glycan synthesis have been identified, and mutant cell lines defective in this enzymatic  
20 pathway have been isolated including the Chinese hamster ovary (CHO) cell lines Lec23 (defective in alpha-glucosidase I) and Lec18 (novel GlcNAc-TVIII). The glycosylation pattern of peptides produced by these mutant cells is altered relative to normal CHO cells. As discussed herein, the glycosylation defects in these and other mutant cells can be exploited for the purposes of producing a peptide that lacks a complex glycan structure. For  
25 example, peptides produced by Lec23 cells lack sialic acid residues, and thus require less enzymatic manipulation in order to reduce the glycan structure to an elemental trimannosyl core or to Man3GlcNAc4. Thus, peptides produced in these cells can serve as preferred substrates for glycan remodeling. One of ordinary skill in the art could isolate or identify other glycosylation-defective cell lines based on known methods, for example the method  
30 described in Stanley et al., 1990, Somatic Cell Mol. Genet., 16: 211-223. Use of glycosylation-defective cell lines, those identified and as yet unidentified, is included in the

invention for the purpose of generating preferred peptide substrates for the remodeling processes described herein.

Expression vectors useful for expressing exogenous peptides in mammalian cells are numerous, and are well known to those in the art. Many mammalian expression vectors are now commercially available from companies, including Novagen, Inc (Madison, WI), Gene Therapy Systems (San Diego, CA), Promega (Madison, WI), ClonTech Inc. (Palo Alto, CA), and Stratagene (La Jolla, CA), among others.

There are several mammalian cell lines that are particularly adept at expressing exogenous peptides. Typically mammalian cell lines originate from tumor cells extracted from mammals that have become immortalized, that is to say, they can replicate in culture essentially indefinitely. These cell lines include, but are not limited to, CHO (Chinese hamster ovary, e.g. CHO-K1; ATCC No. CCL 61) and variants thereof, NS0 (mouse myeloma), BNK, BHK 570 (ATCC No. CRL 10314), BHK (ATCC No. CRL 1632), Per.C6™ (immortalized human cells, Crucell N.V., Leiden, The Netherlands), COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), HEK 293, mouse L cells, T lymphoid cell lines, BW5147 cells and MDCK (Madin-Darby canine kidney), HeLa (human), A549 (human lung carcinoma), 293 (ATCC No. CRL 1573; Graham et al., 1977, Gen. Virol. 36:59-72), BGMK (Buffalo Green Monkey kidney), Hep-2 (human epidermoid larynx carcinoma), LLC-MK<sub>2</sub> (African Green Monkey Kidney), McCoy, NCI-H292 (human pulmonary mucoepidermoid carcinoma tube), RD (rhabdomyosarcoma), Vero (African Green Monkey kidney), HEL (human embryonic lung), Human Fetal Lung-Chang, MRC5 (human embryonic lung), MRHF (human foreskin), and WI-38 (human embryonic lung). In some cases, the cells in which the therapeutic peptide is expressed may be cells derived from the patient to be treated, or they may be derived from another related or unrelated mammal. For example, fibroblast cells may be isolated from the mammal's skin tissue, and cultured and transformed *in vitro*. This technology is commercially available from Transkaryotic Therapies, Inc. (Cambridge, MA). Almost all currently used cell lines are available from the American Type Culture Collection (ATCC, Manassas, VA) and BioWhittaker (Walkersville, Maryland).

Mammalian cells may be transformed with DNA using any one of several techniques that are well known to those in the art. Such techniques include, but are not limited to,

calcium phosphate transformation (Chen and Okayama, 1988 ; Graham and van der Eb, 1973; Corsaro and Pearson, 1981, Somatic Cell Genetics 7:603), Diethylaminoethyl (DEAE)-dextran transfection (Fujita et al., 1986; Lopata et al., 1984; Selden et al., 1986, ), electroporation (Neumann et al., 1982, ; Potter, 1988, ; Potter et al., 1984, ; Wong and Neuman, 1982 ), cationic lipid reagent transfection (Elroy-Stein and Moss, 1990; Feigner et al., 1987; Rose et al., 1991; Whitt et al., 1990; Hawley-Nelson et al., 1993, Focus 15:73; Ciccarone et al., 1993, Focus 15:80), retroviral (Cepko et al., 1984; Miller and Baltimore, 1986; Pear et al., 1993; Austin and Cepko, 1990; Bodine et al., 1991; Fekete and Cepko, 1993; Lemischka et al., 1986; Turner et al., 1990; Williams et al., 1984; Miller and Rosman, 1989, BioTechniques 7:980-90; Wang and Finer, 1996, Nature Med. 2:714-6), polybrene (Chaney et al, 1986; Kawai and Nishizawa, 1984), microinjection (Capecchi, 1980), and protoplast fusion (Rassoulzadegan et al., 1982; Sandri-Goldin et al., 1981; Schaffer, 1980), among others. In general, see Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York) and Ausubel et al. (2002, Current Protocols in Molecular Biology, John Wiley & Sons, New York) for transformation techniques.

Recently the baculovirus system, popular for transformation of insect cells, has been adapted for stable transformation of mammalian cells (see, for review, Koat and Condreay, 2002, Trends Biotechnol. 20:173-180, and references cited therein). The production of recombinant peptides in cultured mammalian cells is disclosed, for example, in U.S. Pat. Nos. 4,713,339, 4,784,950; 4,579,821; and 4,656,134. Several companies offer the services of transformation and culture of mammalian cells, including Cell Trends, Inc. (Middletown, MD). Techniques for culturing mammalian cells are well known in the art, and further found in Hauser et al. (1997, Mammalian Cell Biotechnology, Walter de Gruyter, Inc., Hawthorne, NY), and Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor and references cited therein.

#### D. Insect

Insect cells and in particular, cultured insect cells, express peptides having N-linked glycan structures that are rarely sialylated and usually comprise mannose residues which may or may not have additional fucose residues attached thereto. Examples of the types of glycan structures present on peptides produced in cultured insect cells are shown in Figure 6, and

mannose glycans thereof. In this situation, there may or may not be a core fucose present, which if present, may be linked to the glycan via several different linkages.

Baculovirus-mediated expression in insect cells has become particularly well-established for the production of recombinant peptides (Altmann et al., 1999, Glycoconjugate J. 16:109-123). With regard to peptide folding and post-translational processing, insect cells are second only to mammalian cell lines. However, as noted above, N-glycosylation of peptides in insect cells differs in many respects from N-glycosylation in mammalian cells particularly in that insect cells frequently generate truncated glycan structures comprising oligosaccharides containing just three or sometimes only two mannose residues. These structures may be additionally substituted with fucose residues.

According to the present invention, a peptide produced in an insect cell may be remodeled *in vitro* to generate a peptide with desired glycosylation by first optionally removing any substituted fucose residues using an appropriate fucosidase enzyme. In instances where the peptide comprises an elemental trimannosyl core structure following the removal of fucose residues, then all that is required is the *in vitro* addition of the appropriate sugars to the trimannosyl core structure to generate a peptide having desired glycosylation. In instances when the peptide might contain only two mannose residues in the glycan structure following removal of any fucose residues, a third mannose residue may be added using a mannosyltransferase enzyme and a suitable donor molecule such as GDP-mannose, and thereafter the appropriate residues are added to generate a peptide having desired glycosylation. Optionally, monoantennary glycans can also be generated from these species.

Protocols for the use of baculovirus to transform insect cells are well known to those in the art. Several books have been published which provide the procedures to use the baculovirus system to express peptides in insect cells. These books include, but are not limited to, Richardson (Baculovirus Expression Protocols, 1998, Methods in Molecular Biology, Vol 39, Humana Pr), O'Reilly et al. (1994, Baculovirus Expression Vectors : A Laboratory Manual, Oxford Univ Press), and King and Possee (1992, The Baculovirus Expression System : A Laboratory Guide, Chapman & Hall). In addition, there are also publications such as Lucklow (1993, Curr. Opin. Biotechnol. 4:564-572) and Miller (1993, Curr. Opin. Genet. Dev. 3:97-101).

Many patents have also been issued that related to systems for baculoviral expression of foreign proteins. These patents include, but are not limited to, U.S. Patent No. 6,210,966 (Culture medium for insect cells lacking glutamine and containing ammonium salt), U.S. Patent No. 6,090,584 (Use of BVACs (BaculoVirus Artificial Chromosomes) to produce recombinant peptides), U.S. Patent No. 5,871,986 (Use of a baculovirus to express a recombinant nucleic acid in a mammalian cell), U.S. Patent No. 5,759,809 (Methods of expressing peptides in insect cells and methods of killing insects), U.S. Patent No. 5,753,220 (Cysteine protease gene defective baculovirus, process for its production, and process for the production of economic peptide by using the same), U.S. Patent No. 5,750,383 (Baculovirus cloning system), U.S. Patent No. 5,731,182 (Non-mammalian DNA virus to express a recombinant nucleic acid in a mammalian cell), U.S. Patent No. 5,728,580 (Methods and culture media for inducing single cell suspension in insect cell lines), U.S. Patent No. 5,583,023 (Modified baculovirus, its preparation process and its application as a gene expression vector), U.S. Patent No. 5,571,709 (Modified baculovirus and baculovirus expression vectors), U.S. Patent No. 5,521,299 (Oligonucleotides for detection of baculovirus infection), U.S. Patent No. 5,516,657 (Baculovirus vectors for expression of secretory and membrane-bound peptides), U.S. Patent No. 5,475,090 (Gene encoding a peptide which enhances virus infection of host insects), U.S. Patent No. 5,472,858 (Production of recombinant peptides in insect larvae), U.S. Patent No. 5,348,886 (Method of producing recombinant eukaryotic viruses in bacteria), U.S. Patent No. 5,322,774 (Prokaryotic leader sequence in recombinant baculovirus expression system), U.S. Patent No. 5,278,050 (Method to improve the efficiency of processing and secretion of recombinant genes in insect systems), U.S. Patent No. 5,244,805 (Baculovirus expression vectors), U.S. Patent No. 5,229,293 (Recombinant baculovirus), U.S. Patent No. 5,194,376 (Baculovirus expression system capable of producing recombinant peptides at high levels), U.S. Patent No. 5,179,007 (Method and vector for the purification of recombinant peptides), U.S. Patent No. 5,169,784 (Baculovirus dual promoter expression vector), U.S. Patent No. 5,162,222 (Use of baculovirus early promoters for expression of recombinant nucleic acids in stably transformed insect cells or recombinant baculoviruses), U.S. Patent No. 5,155,037 (Insect signal sequences useful to improve the efficiency of processing and secretion of recombinant nucleic acids in insect systems), U.S. Patent No. 5,147,788 (Baculovirus vectors and methods

of use), U.S. Patent No. 5,110,729 (Method of producing peptides using baculovirus vectors in cultured cells), U.S. Patent No. 5,077,214 (Use of baculovirus early promoters for expression of recombinant genes in stably transformed insect cells), U.S. Patent No. 5,023,328 (Lepidopteran AKH signal sequence), and U.S. Patent Nos. 4,879,236 and 4,745,051 (Method for producing a recombinant baculovirus expression vector). All of the  
5      aforementioned patents are incorporated in their entirety by reference herein.

Insect cell lines of several different species origin are currently being used for peptide expression, and these lines are well known to those in the art. Insect cell lines of interest include, but are not limited to, dipteran and lepidopteran insect cells in general, Sf9 and  
10      variants thereof (fall armyworm *Spodoptera frugiperda*), *Estigmene acrea*, *Trichoplusia ni*, *Bombyx mori*, *Malacosoma disstri*. drosophila lines Kc1 and SL2 among others, and mosquito.

#### E. Plants

Plant cells as peptide producers present a different set of issues. While N-linked  
15      glycans produced in plants comprise a trimannosyl core structure, this pentasaccharide backbone may comprise several different additional sugars as shown in Figure 5. For example, in one instance, the trimannosyl core structure is substituted by a  $\beta$ 1,2 linked xylose residue and an  $\alpha$ 1,3 linked fucose residue. In addition, plant cells may also produce a  
20      Man5GlcNAc2 structure. Peptides produced in plant cells are often highly antigenic as a result of the presence of the core  $\alpha$ 1,3 fucose and xylose on the glycan structure, and are rapidly cleared from the blood stream when introduced into a mammal due to the absence of terminal sialic acid residues. Therefore, unless these peptides are remodeled using the  
25      methods provided herein, they are generally considered to be unsuitable as therapeutic agents in mammals. While some monoclonal antibodies expressed in plant cells were found to be non-immunogenic in mouse, it is likely that the glycan chains were not immunogenic because they were buried in the Fc region in these antibodies (Chargelegue et al., 2000, Transgenic Res. 9(3):187-194).

Following the directions provided herein, it is now possible to generate a peptide  
30      produced in a plant cell wherein an increased number of the glycan structures present thereon comprise an elemental trimannosyl core structure, or a Man3GlcNAc4 structure. This is

accomplished by cleaving off any additional sugars *in vitro* using a combination of appropriate glycosidases, including fucosidases, until the elemental trimannosyl core structure or the Man3GlcNAc4 structure is arrived at. These cleavage reactions should also include removal of any fucose or xylose residues from the structures in order to diminish the antigenicity of the final peptide when introduced into a mammal. Plant cells having mutations that inhibit the addition of fucose and xylose residues to the trimannosyl core structure are known in the art (von Schaewen et al., 1993, Plant Physiology 102:1109-1118). The use of these cells to produce peptides having glycans which lack fucose and xylose is contemplated by the invention. Upon production of the elemental trimannosyl core or Man3GlcNAc4 structure, additional sugars may then be added thereto to arrive at a peptide having desired glycosylation that is therefore suitable for therapeutic use in a mammal.

Transgenic plants are considered by many to be the expression system of choice for pharmaceutical peptides. Potentially, plants can provide a cheaper source of recombinant peptides. It has been estimated that the production costs of recombinant peptides in plants could be between 10 to 50 times lower than that of producing the same peptide in *E. coli*. While there are slight differences in the codon usage in plants as compared to animals, these can be compensated for by adjusting the recombinant DNA sequences (see, Kusnadi et al., 1997, Biotechnol. Bioeng. 56:473-484; Khoudi et al., 1999, Biotechnol. Bioeng. 135-143; Hood et al., 1999, Adv. Exp. Med. Biol. 464:127-147). In addition, peptide synthesis, secretion and post-translational modification are very similar in plants and animals, with only minor differences in plant glycosylation (see, Fischer et al., 2000, J. Biol. Regul. Homest. Agents 14: 83-92). Then, products from transgenic plants are also less likely to be contaminated by animal pathogens, microbial toxins and oncogenic sequences.

The expression of recombinant peptides in plant cells is well known in the art. In addition to transgenic plants, peptides can also be produced in transgenic plant cell cultures (Lee et al., 1997, Mol. Cell. 7:783-787), and non-transgenic plants inoculated with recombinant plant viruses. Several books have been published that describe protocols for the genetic transformation of plant cells: Potrykus (1995, Gene transfer to plants, Springer, New York), Nickoloff (1995, Plant cell electroporation and electrofusion protocols, Humana Press, Totowa, New York) and Draper (1988, Plant genetic transformation, Oxford Press, Boston).



Several methods are currently used to stably transform plant cells with recombinant genetic material. These methods include, but are not limited to, *Agrobacterium* transformation (Bechtold and Pelletier, 1998; Escudero and Hohn, 1997; Hansen and Chilton, 1999; Touraev et al., 1997), biolistics (microprojectiles) (Finer et al., 1999; Hansen and Chilton, 1999; Shilito, 1999), electroporation of protoplasts (Fromm et al., 1985, Ou-Lee et al., 1986; Rhodes et al., 1988; Saunders et al., 1989; Trick et al., 1997), polyethylene glycol treatment (Shilito, 1999; Trick et al., 1997), *in planta* microinjection (Leduc et al., 1996; Zhou et al., 1983), seed imbibition (Trick et al., 1997), laser beam (1996), and silicon carbide whiskers (Thompson et al., 1995; U.S. Patent Appln. No. 20020100077, incorporated by reference herein in its entirety).

Many kinds of plants are amenable to transformation and expression of exogenous peptides. Plants of particular interest to express the peptides to be used in the remodeling method of the invention include, but are not limited to, *Arabidopsis thaliana*, rapeseed (*Brassica* spp.; Ruiz and Blumwald, 2002, *Planta* 214:965-969), soybean (*Glycine max*), sunflower (*Helianthus annuus*), oil palm (*Elaeis guineensis*), groundnut (peanut, *Arachis hypogaea*; Deng et al., 2001, *Cell. Res.* 11:156-160), coconut (*Cocos nucifera*), castor (*Ricinus communis*), safflower (*Carthamus tinctorius*), mustard (*Brassica* spp. and *Sinapis alba*), coriander, (*Coriandrum sativum*), squash (*Cucurbita maxima*; Spencer and Snow, 2001, *Heredity* 86(Pt 6):694-702), linseed/flax (*Linum usitatissimum*; Lamblin et al., 2001, *Physiol Plant* 112:223-232), Brazil nut (*Bertholletia excelsa*), jojoba (*Simmondsia chinensis*), maize (*Zea mays*; Hood et al., 1999, *Adv. Exp. Med. Biol.* 464:127-147; Hood et al., 1997, *Mol. Breed.* 3:291-306; Petolino et al., 2000, *Transgenic Research* 9:1-9), alfalfa (Khouidi et al., 1999, *Biotechnol. Bioeng.* 64:135-143), tobacco (*Nicotiana tabacum*; Wright et al., 2000, *Transgenic Res.* 10:177-181; Frigerio et al., 2000, *Plant Physiol.* 123:1483-1493; Cramer et al., 1996, *Ann. New York Acad. Sci.* 792:62-8-71; Cabanes-Macheteau et al., 1999, *Glycobiology* 9:365-372; Ruggiero et al., 2000, *FEBS Lett.* 469:132-136), canola (Bai et al., 2001, *Biotechnol. Prog.* 17:168-174; Zhang et al., 2000, *J. Anim. Sci.* 78:2868-2878), potato (Tacket et al., 1998, *J. Infect. Dis.* 182:302-305; Richter et al., 2000, *Nat. Biotechnol.* 18:1167-1171; Chong et al., 2000, *Transgenic Res.* 9:71-78), alfalfa (Wigdorovitz et al., 1999, *Virology* 255:347-353), Pea (*Pisum sativum*; Perrin et al., 2000, *Mol. Breed.* 6:345-352), rice (*Oryza sativa*; Stoger et al., 2000, *Plant Mol. Biol.* 42:583-590), cotton

(*Gossypium hirsutum*; Kornyejev et al., 2001, *Physiol Plant* 113:323-331), barley (*Hordeum vulgare*; Petersen et al., 2002, *Plant Mol Biol* 49:45-58); wheat (*Triticum* spp.; Pellegrineschi et al., 2002, *Genome* 45:421-430) and bean (*Vicia* spp.; Saalbach et al., 1994, *Mol Gen Genet* 242:226-236).

5        If expression of the recombinant nucleic acid is desired in a whole plant rather than in cultured cells, plant cells are first transformed with DNA encoding the peptide, following which, the plant is regenerated. This involves tissue culture procedures that are typically optimized for each plant species. Protocols to regenerate plants are already well known in the art for many species. Furthermore, protocols for other species can be developed by one of  
10        skill in the art using routine experimentation. Numerous laboratory manuals are available that describe procedures for plant regeneration, including but not limited to, Smith (2000, *Plant tissue culture : techniques and experiments*, Academic Press, San Diego), Bhojwani and Razdan (1996, *Plant tissue culture : theory and practice*, Elsevier Science Pub., Amsterdam), Islam (1996, *Plant tissue culture*, Oxford & IBH Pub. Co., New Delhi, India), Dodds and  
15        Roberts ( 1995, *Experiments in plant tissue culture*, New York : Cambridge University Press, Cambridge England), Bhojwani (*Plant tissue culture : applications and limitations*, Elsevier, Amsterdam, 1990), Trigiano and Gray (2000, *Plant tissue culture concepts and laboratory exercises*, CRC Press, Boca Raton, Fla), and Lindsey (1991, *Plant tissue culture manual : fundamentals and applications*, Kluwer Academic, Boston).

20        While purifying recombinant peptides from plants may potentially be costly, several systems have been developed to minimize these costs. One method directs the synthesized peptide to the seed endosperm from where it can easily be extracted (Wright et al., 2001, *Transgenic Res.* 10:177-181, Guda et al., 2000, *Plant Cell Res.* 19:257-262; and U.S. Patent No. 5,767,379, which is incorporated by reference herein in its entirety). An alternative  
25        approach is the co-extraction of the recombinant peptide with conventional plant products such as starch, meal or oil. In oil-seed rape, a fusion peptide of oleosin-hurudin when expressed in the plant, attaches to the oil body of the seed, and can be extracted from the plant seed along with the oil (Parmenter, 1995, *Plant Mol. Biol.* 29:1167-1180; U.S. Patent Nos. 5,650,554, 5,792,922, 5,948,682 and 6,288,304, and US application 2002/0037303, all  
30        of which are incorporated in their entirety by reference herein). In a variation on this approach, the oleosin is fused to a peptide having affinity for the exogenous co-expressed

peptide of interest (U.S. Patent No. 5,856,452, incorporated by reference herein in its entirety).

Expression of recombinant peptides in plant plastids, such as the chloroplast, generates peptides having no glycan structures attached thereto, similar to the situation in prokaryotes. However, the yield of such peptides is vastly greater when expressed in these plant cell organelles, and thus this type of expression system may have advantages over other systems. For a general review on the technology for plastid expression of exogenous peptides in higher plants, see Hager and Beck (2000, Appl. Microbiol. Biotechnol. 54:302-310, and references cited therein). Plastid expression has been particularly successful in tobacco (see, for example, Staub et al., 2000, Nat. Biotechnol. 18:333-338).

#### F. Transgenic animals

Introduction of a recombinant DNA into the fertilized egg of an animal (e.g., a mammal) may be accomplished using any number of standard techniques in transgenic animal technology. See, e.g., Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986; and U.S. Pat. No. 5,811,634, which is incorporated by reference herein in its entirety. Most commonly, the recombinant DNA is introduced into the embryo by way of pronuclear microinjection (Gordon et al., 1980, PNAS 77:7380-7384; Gordon and Ruddle, 1981, Science 214:1244-1246; Brinster et al., 1981, Cell 27:223-231; Costantini and Lacy, 1981, Nature 294:92-94). Microinjection has the advantage of being applicable to a wide variety of species. Preimplantation embryos may also be transformed with retroviruses (Jaenisch and Mintz, 1974, Proc. Natl. Acad. Sci. U.S.A. 71:1250-1254; Jaenisch et al., 1976, Hamatol Bluttransfus. 19:341-356; Stuhlmann et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7151-7155). Retroviral mediated transformation has the advantage of adding single copies of the recombinant nucleic acid to the cell, but it produces a high degree of mosaicism. Most recently, embryonic stem cell-mediated techniques have been used (Gossler et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:9065-9069), transfer of entire chromosomal segments (Lavitrano et al., 1989, Cell 57:717-723), and gamete transfection in conjunction with *in vitro* fertilization (Lavitrano et al., 1989, Cell 57:717-723) have also been used. Several books of laboratory procedures have been published disclosing these techniques: Cid-Arregui and García-Carrancá (1998, Microinjection and Transgenesis : Strategies and Protocols, Springer,

Berlin), Clarke (2002, Transgenesis Techniques : Principles and Protocols, Humana Press, Totowa, NJ), and Pinkert (1994, Transgenic Animal Technology : A Laboratory Handbook, Academic Press, San Diego).

Once the recombinant DNA is introduced into the egg, the egg is incubated for a short period of time and is then transferred into a pseudopregnant animal of the same species from which the egg was obtained (Hogan et al., supra). In the case of mammals, typically 125 eggs are injected per experiment, approximately two-thirds of which will survive the procedure. Twenty viable eggs are transferred into a pseudopregnant mammal, four to ten of which will develop into live progeny. Typically, 10-30% of the progeny (in the case of mice) carry the recombinant DNA.

While the entire animal can be used as an expression system for the peptides of the invention, in a preferred embodiment, the exogenous peptide accumulates in products of the animal, from which it can be harvested without injury to the animal. In preferred embodiments, the exogenous peptide accumulates in milk, eggs, hair, blood, and urine.

If the recombinant peptide is to be accumulated in the milk of the animal, suitable mammals are ruminants, ungulates, domesticated mammals, and dairy animals. Particularly preferred animals are goats, sheep, camels, cows, pigs, horses, oxen, and llamas. Methods for generating transgenic cows that accumulate a recombinant peptide in their milk are well known: see, Newton (1999, J. Immunol. Methods 231:159-167), Ebert et al. (1991, Biotechnology 9: 835-838), and U.S. Patent Nos. 6,210,736, 5,849,992, 5,843,705, 5,827,690, 6,222,094, all of which are incorporated herein by reference in their entirety. The generation of transgenic mammals that produce a desired recombinant peptide is commercially available from GTC Biotherapeutics, Framingham, MA.

If the recombinant peptide is to be accumulated in eggs, suitable birds include, but are not limited to, chickens, geese, and turkeys. Other animals of interest include, but are not limited to, other species of avians, fish, reptiles and amphibians. The introduction of recombinant DNA to a chicken by retroviral transformation is well known in the art: Thoraval et al. (1995, Transgenic Research 4:369-376), Bosselman et al., (1989, Science 243: 533-535), Petropoulos et al. (1992, J. Virol. 66: 3391-3397), U.S. Patent No. 5,162,215, incorporated by reference herein in its entirety. Successful transformation of chickens with recombinant DNA also been achieved wherein DNA is introduced into blastodermal cells

and blastodermal cells so transfected are introduced into the embryo: Brazolot et al. (1991, Mol. Reprod. Dev. 30: 304-312), Fraser, et al. (1993, Int. J. Dev. Biol. 37: 381-385), and Petite et al. (1990, Development 108: 185-189). High throughput technology has been developed to assess whether a transgenic chicken expresses the desired peptide (Harvey et al., 2002, Poult. Sci. 81:202-212, U.S. Patent No. 6,423,488, incorporated by reference herein in its entirety). Using retroviral transformation of chicken with a recombinant DNA, exogenous beta-lactamase was accumulated in the egg white of the chicken (Harvey et al., 2002, Nat. Biotechnol. 20(4):396-399). The production of chickens producing exogenous peptides in egg is commercially available from AviGenics, Inc., Athens GA.

#### G. Bacteria

Recombinantly expressed peptides produced in bacteria are not generally glycosylated. However, bacteria systems capable of glycosylating peptides are becoming evident and therefore it is likely that glycosylated recombinant peptides may be produced in bacteria in the future.

Numerous bacterial expression systems are known in the art. Preferred bacterial species include, but are not limited to, *E.coli*. and *Bacillus* species.

The expression of recombinant peptides in *E. coli* is well known in the art. Protocols for *E. coli*-based expression systems are found in U.S. Appln No. 20020064835, U.S. Patent Nos. 6,245,539, 5,606,031, 5,420,027, 5,151,511, and RE33,653, among others. Methods to transform bacteria include, but are not limited to, calcium chloride (Cohen et al., 1972, Proc. Natl. Acad. Sci. U.S.A.. 69:2110-2114; Hanahan, 1983, J. Mol. Biol. 166:557-580; Mandel and Higa, 1970, J. Mol. Biol. 53:159-162) and electroporation (Shigekawa and Dower, 1988, Biotechniques 6:742-751), and those described in Sambrook et al., 2001 (supra). For a review of laboratory protocols on microbial transformation and expression systems, see Saunders and Saunders (1987, Microbial Genetics Applied to Biotechnology : Principles and Techniques of Gene Transfer and Manipulation, Croom Helm, London), Pühler (1993, Genetic Engineering of Microorganisms, Weinheim, New York), Lee et al., (1999, Metabolic Engineering, Marcel Dekker, New York), Adolph (1996, Microbial Genome Methods, CRC Press, Boca Raton), and Birren and Lai (1996, Nonmammalian Genomic Analysis : A Practical Guide, Academic Press, San Diego),

For a general review on the literature for peptide expression in *E. coli* see Balbas (2001, Mol. Biotechnol. 19:251-267). Several companies now offer bacterial strains selected for the expression of mammalian peptides, such as the Rosetta™ strains of *E. coli* (Novagen, inc., Madison, WI; with enhanced expression of eukaryotic codons not normally used in bacteria cells, and enhanced disulfide bond formation),

#### H. Cell engineering

It will be apparent from the present disclosure that the more uniform the starting material produced by a cell, the more efficient will be the generation *in vitro* of large quantities of peptides having desired glycosylation. Thus, the genetic engineering of host cells to produce uniformly glycosylated peptides as starting material for the *in vitro* enzymatic reactions disclosed herein, provides a significant advantage over using a peptide starting material having a heterogeneous set of glycan structures attached thereto. One preferred peptide starting material for use in the present invention is a peptide having primarily glycan molecules which consist solely of an elemental trimannosyl core structure. Another preferred starting material is Man3GlcNAc4. Following the remodeling process, the preferred peptides will give rise to the greatest amount of peptides having desired glycosylation, and thus improved clinical efficacy. However, other glycan starting material is also suitable for use in the methods described herein, in that for example, high mannose glycans may be easily reduced, *in vitro*, to elemental trimannosyl core structures using a series of mannosidases. As described elsewhere herein, other glycan starting material may also be used, provided it is possible to cleave off all extraneous sugar moieties so that the elemental trimannosyl core structure or Man3GlcNAc4 is generated. Thus, the purpose of using genetically engineered cells for the production of the peptides of the present invention is to generate peptides having as uniform as possible a glycan structure attached thereto, wherein the glycan structure can be remodeled *in vitro* to generate a peptide having desired glycosylation. This will result in a dramatic reduction in production costs of these peptides. Since the glycopeptides produced using this methodology will predominantly have the same N-linked glycan structure, the post-production modification protocol can be standardized and optimized to produce a greater batch-to-batch consistency of final product. As a result, the final completed-chain products may be less heterogeneous than those presently available. The products will have an improved biological half-life and bioactivity as compared to the

products of the prior art. Alternatively, if desired, the invention can be used to introduce limited and specific heterogeneity, e.g., by choosing reaction conditions that result in differential addition of sugar moieties.

Preferably, though not as a rigid requirement, the genetically engineered cell is one which produces peptides having glycan structures comprised primarily of an elemental trimannosyl core structure or Man3GlcNAc4. At a minimum, the proportion of these preferred structures produced by the genetically engineered cell must be enough to yield a peptide having desired glycosylation following the remodeling protocol.

In general, any eukaryotic cell type can be modified to become a host cell of the present invention. First, the glycosylation pattern of both endogenous and recombinant glycopeptides produced by the organism are determined in order to identify suitable additions/deletions of enzymatic activities that result in the production of elemental trimannosyl core glycopeptides or Man3GlcNAc4 glycopeptides. This will typically entail deleting activities that use trimannosyl glycopeptides as substrates for a glycosyltransferase reaction and inserting enzymatic activities that degrade more complex N-linked glycans to produce shorter chains. In addition, genetically engineered cells may produce high mannose glycans, which may be cleaved by mannosidase to produce desired starting glycan structures. The mannosidase may be active *in vivo* in the cell (i.e., the cell may be genetically engineered to produce them), or they may be used in *in vitro* post production reactions.

Techniques for genetically modifying host cells to alter the glycosylation profile of expressed peptides are well-known. See, e.g., Altmann et al. (1999, Glycoconjugate J. 16: 109-123), Ailor et al. (2000, Glycobiology 10(8): 837-847), Jarvis et al., (*In vitro*gen Conference, March, 1999, abstract), Hollister and Jarvis, (2001, Glycobiology 11(1): 1-9), and Palacpac et al., (1999, PNAS USA 96: 4697), Jarvis et al., (1998, Curr. Opin. Biotechnol. 9:528-533), Gerngross (U.S. Patent Publication No. 20020137134), all of which disclose techniques to "mammalianize" insect or plant cell expression systems by transfecting insect or plant cells with glycosyltransferase genes.

Techniques also exist to genetically alter the glycosylation profile of peptides expressed in *E. coli*. *E. coli* has been engineered with various glycosyltransferases from the bacteria *Neisseria meningitidis* and *Azorhizobium* to produce oligosaccharides *in vivo* (Bettler et al., 1999, Glycoconj. J. 16:205-212). *E. coli* which has been genetically engineered to

over-express *Neisseria meningitidis*  $\beta$ 1,3 N acetyl glucosaminyltransferase lgtA gene will efficiently glycosylate exogenous lactose (Priem et al., 2002, Glycobiology 12:235-240).

Fungal cells have also been genetically modified to produce exogenous glycosyltransferases (Yoshida et al., 1999, Glycobiology, 9(1):53-58; Kalsner et al., 1995, Glycoconj. J. 12:360-370; Schwientek and Ernst, 1994, Gene 145(2):299-303; Chiba et al., 1995, Biochem J. 308:405-409).

Thus, in one aspect, the present invention provides a cell that glycosylates a glycopeptide population such that a proportion of glycopeptides produced thereby have an elemental trimannosyl core or a Man3GlcNAc4 structure. Preferably, the cell produces a peptide having a glycan structure comprised solely of an elemental trimannosyl core. At a minimum, the proportion of peptides having an elemental trimannosyl core or a Man3GlcNAc4 structure is enough to yield peptides having desired glycosylation following the remodeling process. The cell has introduced into it one or more heterologous nucleic acid expression units, each of which may comprise one or more nucleic acid sequences encoding one or more peptides of interest. The natural form of the glycopeptide of interest may comprise one or more complex N-linked glycans or may simply be a high mannose glycan.

The cell may be any type of cell and is preferably a eukaryotic cell. The cell may be a mammalian cell such as human, mouse, rat, rabbit, hamster or other type of mammalian cell. When the cell is a mammalian cell, the mammalian cell may be derived from or contained within a non-human transgenic mammal where the cell in the mammal encodes the desired glycopeptide and a variety of glycosylating and glycosidase enzymes as necessary for the production of desired glycopeptide molecules. In addition, the cell may be a fungal cell, preferably, a yeast cell, or the cell may be an insect or a plant cell. Similarly, when the cell is a plant cell, the plant cell may be derived from or contained within a transgenic plant, wherein the plant encodes the desired glycopeptide and a variety of glycosylating and glycosidase enzymes as are necessary for the production of desired glycopeptide molecules.

In some embodiments the host cell may be a eukaryotic cell expressing one or more heterologous glycosyltransferase enzymes and/or one or more heterologous glycosidase enzymes, wherein expression of a recombinant glycopeptide in the host cell results in the production of a recombinant glycopeptide having an elemental trimannosyl core as the primary glycan structure attached thereto.



In some embodiments the heterologous glycosyltransferase enzyme useful in the cell may be selected from a group consisting of any known glycosyltransferase enzyme included for example, in the list of Glycosyltransferase Families available in Taniguchi et al. (2002, Handbook of Glycosyltransferases and Related Genes, Springer, New York).

5 In other embodiments, the heterologous glycosylase enzyme may be selected from a group consisting of mannosidase 1, mannosidase 2, mannosidase 3, and other mannosidases, including, but not limited to, microbial mannosidases. Additional disclosure regarding enzymes useful in the present invention is provided elsewhere herein.

10 In yet other embodiments, the host cell may be a eukaryotic cell wherein one or more endogenous glycosyltransferase enzymes and/or one or more endogenous glycosidase enzymes have been inactivated such that expression of a recombinant glycopeptide in the host cell results in the production of a recombinant glycopeptide having an elemental trimannosyl core as the primary glycan structure attached thereto.

15 In additional embodiments, the host cell may express heterologous glycosyltransferase enzymes and/or glycosidase enzymes while at the same time one or more endogenous glycosyltransferase enzymes and/or glycosidase enzymes are inactivated. Endogenous glycosyltransferase enzymes and/or glycosidase enzymes may be inactivated using any technique known to those skilled in the art including, but not limited to, antisense techniques and techniques involving insertion of nucleic acids into the genome of the host  
20 cell. In some embodiments, the endogenous enzymes may be selected from a group consisting of GnT-I, a selection of mannosidases, xylosyltransferase, core  $\alpha$ 1,3 fucosyltransferase, serine/threonine O-mannosyltransferases, and the like.

25 Alternatively, an expression system that naturally glycosylates peptides such that the N-linked glycans are predominantly the trimannosyl core type, or the Man3GlcNAc4 type, can be exploited. An example of a cell type that produces the trimannosyl core is Sf9 cells. Other such expression systems can be identified by analyzing glycopeptides that are naturally or recombinantly expressed in cells and selecting those which exhibit the desired glycosylation characteristics. The invention should be construed to include any and all such cells for the production of the peptides of the present invention.

#### V. Purification of glycan remodeled and/or glycoconjugated peptides

If the modified glycoprotein is produced intracellularly or secreted, as a first step, the particulate debris, either host cells, lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the peptide variant from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (e.g., on diethylaminoethyl (DEAE) or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (RP-HPLC), gel filtration using, e.g., Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the peptide, and ethanol, pH or ammonium sulfate precipitation, membrane filtration and various techniques.

Modified peptides produced in culture are usually isolated by initial extraction from cells, enzymes, etc., followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps. Additionally, the modified glycoprotein may be purified by affinity chromatography. Then, HPLC may be employed for final purification steps.

A protease inhibitor, e.g., phenylmethylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

Within another embodiment, supernatants from systems which produce the modified peptide of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed

in protein purification. Alternatively, a cation-exchange step may be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

Then, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a peptide variant composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous modified glycoprotein.

The modified peptide of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal *et al.*, *J. Chromatog.* **296**: 171 (1984). This reference describes two sequential, RP-HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, techniques such as affinity chromatography may be utilized to purify the modified glycoprotein.

#### VI. Preferred Peptides and Nucleic Acids Encoding Preferred Peptides

The present invention includes isolated nucleic acids encoding various peptides and proteins, and similar molecules or fragments thereof. The invention should not be construed to be limited in any way solely to the use of these peptides in the methods of the invention, but rather should be construed to include any and all peptides presently available or which become available to those in the art. In addition, the invention should not be construed to include only one particular nucleic acid or amino acid sequence for the peptides listed herein, but rather should be construed to include any and all variants, homologs, mutants, etc. of each of the peptides. It should be noted that when a particular peptide is identified as having a mutation or other alteration in the sequence for that peptide, the numbering of the amino acids which identify the alteration or mutation is set so that the first amino acid in the mature peptide sequence is amino acid no. 1, unless otherwise stated herein.

Preferred peptides include, but are not limited to human granulocyte colony stimulating factor (G-CSF), human interferon alpha (IFN-alpha), human interferon beta (IFN-beta), human Factor VII (Factor VII), human Factor IX (Factor IX), human follicle stimulating hormone (FSH), human erythropoietin (EPO), human granulocyte/macrophage colony stimulating factor (GM-CSF), human interferon gamma (IFN-gamma), human alpha-1-protease inhibitor (also known as alpha-1-antitrypsin or alpha-1-trypsin inhibitor; A-1-PI),

glucocerebrosidase, human tissue-type activator (TPA), human interleukin-2 (IL-2), human Factor VIII (Factor VIII), a 75 kDa tumor necrosis factor receptor fused to a human IgG immunoglobulin Fc portion, commercially known as ENBREL™ or ETANERCEPT™ (chimeric TNFR), human urokinase (urokinase), a Fab fragment of the human/mouse  
5 chimeric monoclonal antibody that specifically binds glycoprotein IIb/ IIIa and the vitronectin alpha<sub>v</sub> beta<sub>3</sub> receptor, known commercially as REOPRO™ or ABCIXIMAB (chimeric anti-glycoprotein IIb/IIIa), a mouse/human chimeric monoclonal antibody that specifically binds human HER2, known commercially as HERCEPTIN™ (chimeric anti-HER2), a human/mouse chimeric antibody that specifically binds the A antigenic site or the F  
10 protein of respiratory syncytial virus commercially known as SYNAGIS™ or PALIVIZUMAB (chimeric anti-RSV), a chimeric human/mouse monoclonal antibody that specifically binds CD20 on human B-cells, known commercially as RITUXAN™ or RITUXAMAB (chimeric anti-CD20), human recombinant DNase (DNase), a chimeric human/mouse monoclonal antibody that specifically binds human tumor necrosis factor,  
15 known commercially as REMICADE™ or INFLIXIMAB (chimeric anti-TNF), human insulin, the surface antigen of a hepatitis B virus (adw subtype; HBsAg), and human growth hormone (HGH), alpha-galactosidase A (Fabryzyme™),  $\alpha$ -Iduronidase (Aldurazyme™), antithrombin (antithrombin III, AT-III), human chorionic gonadotropin (hCG), interferon omega, and the like.

20 The isolated nucleic acid of the invention should be construed to include an RNA or a DNA sequence encoding any of the above-identified peptides of the invention, and any modified forms thereof, including chemical modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. As a non-limiting example, oligonucleotides which contain at least one phosphorothioate  
25 modification are known to confer upon the oligonucleotide enhanced resistance to nucleases. Specific examples of modified oligonucleotides include those which contain phosphorothioate, phosphotriester, methyl phosphonate, short chain alkyl or cycloalkyl intersugar linkages, or short chain heteroatomic or heterocyclic intersugar ("backbone") linkages. In addition, oligonucleotides having morpholino backbone structures (U.S. Patent  
30 No: 5,034,506) or polyamide backbone structures (Nielsen et al., 1991, Science 254: 1497) may also be used.

Chemical modifications of nucleotides may also be used to enhance the efficiency with which a nucleotide sequence is taken up by a cell or the efficiency with which it is expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

5       The present invention should not be construed as being limited solely to the nucleic and amino acid sequences disclosed herein. As described in more detail elsewhere herein, once armed with the present invention, it is readily apparent to one skilled in the art that other nucleic acids encoding the peptides of the present invention can be obtained by following the procedures described herein (*e.g.*, site-directed mutagenesis, frame shift mutations, and the  
10   like), and procedures that are well-known in the art.

Also included are isolated nucleic acids encoding fragments of peptides, wherein the peptide fragments retain the desired biological activity of the peptide. In addition, although exemplary nucleic acids encoding preferred peptides are disclosed herein in relation to specific SEQ ID NOS, the invention should in no way be construed to be limited to any  
15   specific nucleic acid disclosed herein. Rather, the invention should be construed to include any and all nucleic acid molecules having a sufficient percent identity with the sequences disclosed herein such that these nucleic acids also encode a peptide having the desired biological activity disclosed herein. Also contemplated are isolated nucleic acids that are shorter than full length nucleic acids, wherein the biological activity of the peptide encoded  
20   thereby is retained. Methods to determine the percent identity between one nucleic acid and another are disclosed elsewhere herein as are assays for the determination of the biological activity of any specific preferred peptide.

Also as disclosed elsewhere herein, any other number of procedures may be used for the generation of derivative, mutant, or variant forms of the peptides of the present invention  
25   using recombinant DNA methodology well known in the art such as, for example, that described in Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, *Current Protocols in Molecular Biology*, Green & Wiley, New York). Procedures for the introduction of amino acid changes in a peptide or polypeptide by altering the DNA sequence encoding the peptide  
30   are well known in the art and are also described in Sambrook et al. (1989, *supra*); Ausubel et al. (1997, *supra*).

The invention includes a nucleic acid encoding a G-CSF, IFN-alpha, IFN-beta, Factor VII, Factor IX, FSH, EPO, GM-CSF, IFN-gamma, A-1-PI, glucocerebrosidase, TPA, IL-2, Factor VIII, chimeric TNFR, urokinase, chimeric anti-glycoprotein IIb/IIa, chimeric anti-HER2, chimeric anti-RSV, chimeric anti-CD20, DNase, chimeric anti-TNF, human insulin, HBsAg, and HGH, wherein a nucleic acid encoding a tag peptide is covalently linked thereto. That is, the invention encompasses a chimeric nucleic acid wherein the nucleic acid sequence encoding a tag peptide is covalently linked to the nucleic acid encoding a peptide of the present invention. Such tag peptides are well known in the art and include, for instance, green fluorescent protein (GFP), myc, myc-pyruvate kinase (myc-PK), His<sub>6</sub>, maltose binding protein (MBP), an influenza virus hemagglutinin tag polypeptide, a flag tag polypeptide (FLAG), and a glutathione-S-transferase (GST) tag polypeptide. However, the invention should in no way be construed to be limited to the nucleic acids encoding the above-listed tag peptides. Rather, any nucleic acid sequence encoding a peptide which may function in a manner substantially similar to these tag peptides should be construed to be included in the present invention.

The nucleic acid comprising a nucleic acid encoding a tag peptide can be used to localize a peptide of the present invention within a cell, a tissue, and/or a whole organism (e.g., a mammalian embryo), detect a peptide of the present invention secreted from a cell, and to study the role(s) of the peptide in a cell. Further, addition of a tag peptide facilitates isolation and purification of the "tagged" peptide such that the peptides of the invention can be produced and purified readily.

The invention includes the following preferred isolated peptides: G-CSF, IFN-alpha, IFN-beta, Factor VII, Factor IX, FSH, EPO, GM-CSF, IFN-gamma, A-1-PI, glucocerebrosidase, TPA, IL-2, Factor VIII, chimeric TNFR, urokinase, chimeric anti-glycoprotein IIb/IIIa, chimeric anti-HER2, chimeric anti-RSV, chimeric anti-CD20, DNase, chimeric anti-TNF, human insulin, HBsAg, HGH, alpha-galactosidase A, ,  $\alpha$ -Iduronidase, antithrombin III, hCG, and interferon omega, and the like.

The present invention should also be construed to encompass "derivatives," "mutants", and "variants" of the peptides of the invention (or of the DNA encoding the same) which derivatives, mutants, and variants are peptides which are altered in one or more amino acids (or, when referring to the nucleotide sequence encoding the same, are altered in one or

more base pairs) such that the resulting peptide (or DNA) is not identical to the sequences recited herein, but has the same biological property as the peptides disclosed herein, in that the peptide has biological/biochemical properties of G-CSF, IFN-alpha, IFN-beta, Factor VII, Factor IX, FSH, EPO, GM-CSF, IFN-gamma, A-1-PI, glucocerebrosidase, TPA, IL-2, Factor  
5 VIII, chimeric TNFR, urokinase, chimeric anti-glycoprotein IIb/IIIa, chimeric anti-HER2, chimeric anti-RSV, chimeric anti-CD20, DNase, chimeric anti-TNF, human insulin, HBsAg, and HGH.

Further included are fragments of peptides that retain the desired biological activity of the peptide irrespective of the length of the peptide. It is well within the skill of the artisan to  
10 isolate smaller than full length forms of any of the peptides useful in the invention, and to determine, using the assays provided herein, which isolated fragments retain a desired biological activity and are therefore useful peptides in the invention.

A biological property of a protein of the present invention should be construed to include, but not be limited to include the ability of the peptide to function in the biological  
15 assay and environments described herein, such as reduction of inflammation, elicitation of an immune response, blood-clotting, increased hematopoietic output, protease inhibition, immune system modulation, binding an antigen, growth, alleviation of treatment of a disease, DNA cleavage, and the like.

#### 20 A. G-CSF

The present invention encompasses a method for the modification of the glycan structure on G-CSF. G-CSF is well known in the art as a cytokine produced by activated T-cells, macrophages, endothelial cells, and stromal fibroblasts. G-CSF primarily acts on the bone marrow to increase the production of inflammatory leukocytes, and further functions as  
25 an endocrine hormone to initiate the replenishment of neutrophils consumed during inflammatory functions. G-CSF also has clinical applications in bone marrow replacement following chemotherapy.

A remodeled G-CSF peptide may be administered to a patient selected from the group consisting of a non-myeloid cancer patient receiving myelosuppressive chemotherapy, a  
30 patient having Acute Myeloid Leukemia (AML) receiving induction or consolidation chemotherapy, a non-myeloid cancer patient receiving a bone marrow transplant, a patient

undergoing peripheral blood progenitor cell collection, a patient having severe chronic neutropenia, and a patient having persistent neutropenia and also having advanced HIV infection. Preferably, the patient is a human patient.

While G-CSF has been shown to be an important and useful compound for therapeutic applications in mammals, especially humans, present methods for the production of G-CSF from recombinant cells results in a product having a relatively short biological life, an inaccurate glycosylation pattern that could potentially lead to immunogenicity, loss of function, and an increased need for both larger and more frequent doses in order to achieve the same effect, and the like.

G-CSF has been isolated and cloned, the nucleic acid and amino acid sequences of which are presented as SEQ ID NO:1 and SEQ ID NO:2, respectively (Figure 58A and 58B, respectively). The present invention encompasses a method for modifying G-CSF, particularly as it relates to the ability of G-CSF to function as a potent and functional biological molecule. The skilled artisan, when equipped with the present disclosure and the teachings herein, will readily understand that the present invention provides compositions and methods for the modification of G-CSF.

The present invention further encompasses G-CSF variants, as well known in the art. As an example, but in no way meant to be limiting to the present invention, a G-CSF variant has been described in U.S. Patent No. 6,166,183, in which a G-CSF comprising the natural complement of lysine residues and further linked to one or two polyethylene glycol molecules is described. Additionally, U.S. Patent Nos. 6,004,548, 5,580,755, 5,582,823, and 5,676,941 describe a G-CSF variant in which one or more of the cysteine residues at position 17, 36, 42, 64, and 74 are replaced by alanine or alternatively serine. U.S. Patent No. 5,416,195 describes a G-CSF molecule in which the cysteine at position 17, the aspartic acid at position 27, and the serines at positions 65 and 66 are substituted with serine, serine, proline, and proline, respectively. Other variants are well known in the art, and are described in, for example, U.S. Patent No. 5,399,345.

The expression and activity of a modified G-CSF molecule of the present invention can be assayed using methods well known in the art, and as described in, for example, U.S. Patent No. 4,810,643. As an example, activity can be measured using radio-labeled thymidine uptake assays. Briefly, human bone marrow from healthy donors is subjected to a



density cut with Ficoll-Hypaque (1.077 g/ml, Pharmacia, Piscataway, NJ) and low density cells are suspended in Iscove's medium (GIBCO, La Jolla, CA) containing 10% fetal bovine serum, glutamine and antibiotics. About  $2 \times 10^4$  human bone marrow cells are incubated with either control medium or the G-CSF or the present invention in 96-well flat bottom plates at about 37° C in 5% CO<sub>2</sub> in air for about 2 days. Cultures are then pulsed for about 4 hours with 0.5 µCi/well of <sup>3</sup>H-thymidine (New England Nuclear, Boston, Mass.) and uptake is measured as described in, for example, Ventua, et al.(1983, Blood 61:781). An increase in <sup>3</sup>H-thymidine incorporation into human bone marrow cells as compared to bone marrow cells treated with a control compound is an indication of a active and viable G-CSF compound.

#### B. IFN alpha, IFN beta and IFN omega

The present invention further encompasses a method for the remodeling and modification of IFN alpha, IFN beta and IFN omega. IFN alpha is part of a family of approximately twenty peptides of approximately 18kDa in weight. IFN omega is very similar in structure and function to IFN alpha. IFN omega is useful for treatment of hepatitis C virus infection when an immune response to IFN alpha is mounted in the host rendering that treatment ineffective. Antibodies raised against IFN alpha do not cross-react with IFN omega. Thus, treatment of hepatitis C may continue using IFN omega when IFN alpha therapy is no longer possible.

IFN alpha, omega, and IFN beta, collectively known as the Type I interferons, bind to the same cellular receptor and elicit similar responses. Type I IFNs inhibit viral replication, increase the lytic potential of NK cells, modulate MHC molecule expression, and inhibit cellular proliferation, among other things. Type I IFN has been used as a therapy for viral infections, particularly hepatitis viruses, and as a therapy for multiple sclerosis.

Current compositions of Type I IFN are, as described above, useful compounds for both the modulation of aberrant immunological responses and as a therapy for a variety of diseases. However, they are hampered by decreased potency and function, and a limited half-life in the body as compared to natural cytokines comprising the natural complement of glycosylation.

A remodeled interferon-alpha peptide may be administered to a patient selected from the group consisting of a patient having hairy cell leukemia, a patient having malignant

melanoma, a patient having follicular lymphoma, a patient having condylomata acuminata, a patient having AIDS-related Kaposi's sarcoma, a patient having Hepatitis C, a patient having Hepatitis B, a patient having a human papilloma virus infection, a patient having Chronic Myeloid Leukemia (CML), a patient having chronic phase Philadelphia chromosome (Ph) positive Chronic Myelogenous Leukemia, a patient having non-Hodgkin's lymphoma (NHL), a patient having lymphoma, a patient having bladder cancer, and a patient having renal cancer. Preferably, the patient is a human patient.

A remodeled interferon-beta peptide may be administered to a patient selected from the group consisting of a patient having multiple sclerosis (MS), a patient having Hepatitis B, a patient having Hepatitis C, a patient having human papilloma virus infection, a patient having breast cancer, a patient having brain cancer, a patient having colorectal cancer, a patient having pulmonary fibrosis, and a patient having rheumatoid arthritis. Preferably, the patient is a human patient.

A remodeled interferon-omega peptide may be administered to a patient selected from the group consisting of a patient having hairy cell leukemia, a patient having malignant melanoma, a patient having follicular lymphoma, a patient having condylomata acuminata, a patient having AIDS-related Kaposi's sarcoma, a patient having Hepatitis C, a patient having Hepatitis B, a patient having a human papilloma virus infection, a patient having Chronic Myeloid Leukemia (CML), a patient having chronic phase Philadelphia chromosome (Ph) positive Chronic Myelogenous Leukemia, a patient having non-Hodgkin's lymphoma (NHL), a patient having lymphoma, a patient having bladder cancer, and a patient having renal cancer. Preferably, the patient is a human patient.

The prototype nucleotide and amino acid sequence for IFN alpha is set forth herein as SEQ ID NO:3 and SEQ ID NO:4, respectively (Figure 59A and 59B, respectively). The prototype nucleotide and amino acid sequence for IFN omega is set forth herein as SEQ ID NO:74 and SEQ ID NO:75, respectively (Figures 59C and 59D, respectively). IFN beta comprises a single gene product of approximately 20 kDa, the nucleic acid and amino acid sequence of which are presented herein as SEQ ID NO:5 and SEQ ID NO:6 (Figure 60A and 60B, respectively). The present invention is not limited to the nucleotide and amino acid sequences herein. One of skill in the art will readily appreciate that many variants of IFN alpha exist both naturally and as engineered derivatives. Similarly, IFN beta has been

modified in attempts to achieve a more beneficial therapeutic profile. Examples of modified Type I IFNs are well known in the art (see Table 9), and are described in, for example U.S. Patent No. 6,323,006, in which cysteine-60 is substituted for tyrosine, U. S. Patent Nos. 4,737,462, 4,588,585, 5,545,723, and 6,127,332 where an IFN beta with a substitution of a variety of amino acids is described. Additionally, U.S. Patent Nos. 4,966,843, 5,376,567, 5,795,779 describe IFN alpha-61 and IFN-alpha-76. U.S. Patent Nos. 4,748,233 and 4,695,543 describe IFN alpha gx-1, whereas U.S. Patent No. 4,975,276 describes IFN alpha-54. In addition, U.S. Patent Nos. 4,695,623, 4,897,471, 5,661,009, and 5,541,293 all describe a consensus IFN alpha sequence to represent all variants known at the date of filing. While this list of Type I IFNs and variants thereof is in no way meant to be exhaustive, one of skill in the art will readily understand that the present invention encompasses IFN beta and IFN alpha molecules, derivatives, and variants known or to be discovered in the future.

Table 9. Interferon- $\alpha$  Isoforms.

$\alpha$ type	AA characteristic
1a	A <sup>114</sup>
1b	V <sup>114</sup>
2a	K <sup>23</sup> -H <sup>34</sup>
2b	R <sup>23</sup> -H <sup>34</sup>
2c	R <sup>23</sup> -R <sup>34</sup>
4a	A <sup>51</sup> -E <sup>114</sup>
4b	T <sup>51</sup> -V <sup>114</sup>
7a	M <sup>132</sup> -K <sup>159</sup> -G <sup>161</sup>
7b	M <sup>132</sup> -Q <sup>159</sup> -R <sup>161</sup>
7c	T <sup>132</sup> -K <sup>159</sup> -G <sup>161</sup>
8a	V <sup>98</sup> -L <sup>99</sup> -C <sup>100</sup> -D <sup>101</sup> -R <sup>161</sup>
8b	S <sup>98</sup> -C <sup>99</sup> -V <sup>100</sup> -M <sup>101</sup> -R <sup>161</sup>
8c	S <sup>98</sup> -C <sup>99</sup> -V <sup>100</sup> -M <sup>101</sup> -D <sup>161</sup> $\Delta$ (162-166)
10a	S <sup>8</sup> -L <sup>89</sup>
10b	T <sup>8</sup> -I <sup>89</sup>
14a	F <sup>152</sup> -Q <sup>159</sup> -R <sup>161</sup>
14b	F <sup>152</sup> -K <sup>159</sup> -G <sup>161</sup>
14c	L <sup>152</sup> -Q <sup>159</sup> -R <sup>161</sup>
17a	P <sup>34</sup> -S <sup>55</sup> -I <sup>161</sup>
17b	H <sup>34</sup> -S <sup>55</sup> -I <sup>161</sup>
17c	H <sup>34</sup> -S <sup>55</sup> -R <sup>161</sup>
17d	H <sup>34</sup> -P <sup>55</sup> -R <sup>161</sup>
21a	M <sup>96</sup>
21b	L <sup>96</sup>

Methods of expressing IFN in recombinant cells are well known in the art, and is easily accomplished using techniques described in, for example U.S. Patent No. 4,966,843, and in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York). Assays to determine the biological activity of a Type I IFN modified by the present invention will be well known to the skilled artisan. For example, the assay described in Rubinstein et al., (1981, Journal of Virology 37:755-758) is commonly used to determine the effect of an Type I IFN by measuring the cytopathic effects of viral infection on a population of cells. This method is only one of many known in the art for assaying the biological function of a Type IFN.

#### C. Factor VIIa

The present invention further encompasses a method for the remodeling and modification of Factor VII. The blood coagulation pathway is a complex reaction comprising many events. An intermediate event in this pathway is Factor VII, a proenzyme that participates in the extrinsic pathway of blood coagulation by converting (upon its activation to Factor VIIa) Factor X to Xa in the presence of tissue factor and calcium ions. Factor Xa in turn then converts prothrombin to thrombin in the presence of Factor Va, calcium ions and phospholipid. The activation of Factor X to Factor Xa is an event shared by both the intrinsic and extrinsic blood coagulation pathways, and therefore, Factor VIIa can be used for the treatment of patients with deficiencies or inhibitors of Factor VIII. There is also evidence to suggest that Factor VIIa may participate in the intrinsic pathway as well therefore increasing the prominence and importance of the role of Factor VII in blood coagulation.

Factor VII is a single-chain glycoprotein with a molecular weight of approximately 50 kDa. In this form, the factor circulates in the blood as an inactive zymogen. Activation of Factor VII to VIIa may be catalyzed by several different plasma proteases, such as Factor XIIa. Activation of Factor VII results in the formation of a heavy chain and a light chain held together by at least one disulfide bond. Further, modified Factor VII molecules that cannot be converted to Factor VIIa have been described, and are useful as anti-coagulation remedies,

such as in the case of blood clots, thrombosis, and the like. Given the importance of Factor VII in the blood coagulation pathway, and its use as a treatment for both increased and decreased levels of coagulation, it follows that a molecule that has a longer biological half-life, increased potency, and in general, a therapeutic profile more similar to wild-type Factor VII as it is synthesized and secreted in the healthy human would be beneficial and useful as a treatment for blood coagulation disorders.

A remodeled Factor VII peptide may be administered to a patient selected from the group consisting of a hemophiliac patient having a bleeding episode, a patient having Hemophilia A, a patient with Hemophilia B, a patient having Hemophilia A, wherein the patient also has antibodies to Factor VIII, a patient having Hemophilia B, wherein the patient also has antibodies to Factor IX, a patient having liver cirrhosis, a cirrhotic patient having an orthotopic liver transplant, a cirrhotic patient having upper gastrointestinal bleeding, a patient having a bone marrow transplant, and a patient having a liver resection. Preferably, the patient is a human patient.

Factor VII has been cloned and sequenced, and the nucleic acid and amino acid sequences are presented herein as SEQ ID NO:7 and SEQ ID NO:8 (Figure 61A and 61B, respectively). The present invention should in no way be construed as limited to the Factor VII nucleic acid and amino acid sequences set forth herein. Variants of Factor VII are described in, for example, U.S. Patent Nos. 4,784,950 and 5,580,560, in which lysine-38, lysine-32, arginine-290, arginine-341, isoleucine-42, tyrosine-278, and tyrosine-332 is replaced by a variety of amino acids. Further, U.S. Patent Nos. 5,861,374, 6,039,944, 5,833,982, 5,788,965, 6,183,743, 5,997,864, and 5,817,788 describe Factor VII variants that are not cleaved to form Factor VIIa. The skilled artisan will recognize that the blood coagulation pathway and the role of Factor VII therein are well known, and therefore many variants, both naturally occurring and engineered, as described above, are included in the present invention.

Methods for the expression and to determine the activity of Factor VII are well known in the art, and are described in, for example, U.S. Patent No. 4,784,950. Briefly, expression of Factor VII, or variants thereof, can be accomplished in a variety of both prokaryotic and eukaryotic systems, including *E. coli*, CHO cells, BHK cells, insect cells using a baculovirus expression system, all of which are well known in the art.

Assays for the activity of a modified Factor VII prepared according to the methods of the present invention can be accomplished using methods well known in the art. As a non-limiting example, Quick et al. (Hemorrhagic Disease and Thrombosis, 2nd ed., Leat Febiger, Philadelphia, 1966), describes a one-stage clotting assay useful for determining the biological activity of a Factor VII molecule prepared according to the methods of the present invention.

#### D. Factor IX

The present invention further encompasses a method for remodeling and/or modifying Factor IX. As described above, Factor IX is vital in the blood coagulation cascade. A deficiency of Factor IX in the body characterizes a type of hemophilia (type B). Treatment of this disease is usually limited to intravenous transfusion of human plasma protein concentrates of Factor IX. However, in addition to the practical disadvantages of time and expense, transfusion of blood concentrates involves the risk of transmission of viral hepatitis, acquired immune deficiency syndrome or thromboembolic diseases to the recipient.

While Factor IX has demonstrated itself as an important and useful compound for therapeutic applications, present methods for the production of Factor IX from recombinant cells (U.S. Patent No. 4,770,999) results in a product with a rather short biological life, an inaccurate glycosylation pattern that could potentially lead to immunogenicity, loss of function, an increased need for both larger and more frequent doses in order to achieve the same effect, and the like.

A remodeled Factor IX peptide may be administered to a patient selected from the group consisting of a hemophiliac patient having a bleeding episode and also having Hemophilia B, a patient having Hemophilia B, a patient having Hemophilia B and having antibodies to Factor IX, a patient having liver cirrhosis, a cirrhotic patient having an orthotopic liver transplant, a cirrhotic patient having upper gastrointestinal bleeding, a patient having a bone marrow transplant, and a patient having a liver resection. A remodeled Factor IX peptide may also be administered to control and/or prevent hemorrhagic episodes in a patient having Hemophilia B, congenital Factor IX deficiency, or Christmas disease. A remodeled Factor IX peptide may also be administered to a patient to control and/or prevent hemorrhagic episodes in the patient during surgery. Preferably, the patient is a human patient.

The nucleic and amino acid sequences of Factor IX is set forth herein as SEQ ID NO:9 and SEQ ID NO:10 (Figure 62A and 62B, respectively). The present invention is in no way limited to the sequences set forth herein. Factor IX variants are well known in the art, as described in, for example, U.S. Patent Nos. 4,770,999, 5,521,070 in which a tyrosine is replaced by an alanine in the first position, U.S. Patent No. 6,037,452, in which Factor XI is linked to an alkylene oxide group, and U.S. Patent No. 6,046,380, in which the DNA encoding Factor IX is modified in at least one splice site. As demonstrated herein, variants of Factor IX are well known in the art, and the present disclosure encompasses those variants known or to be developed or discovered in the future.

Methods for determining the activity of a modified Factor IX prepared according to the methods of the present invention can be carried out using the methods described above, or additionally, using methods well known in the art, such as a one stage activated partial thromboplastin time assay as described in, for example, Biggs (1972, Human Blood Coagulation Haemostasis and Thrombosis (Ed. 1), Oxford, Blackwell, Scientific, pg. 614). Briefly, to assay the biological activity of a Factor IX molecule developed according to the methods of the present invention, the assay can be performed with equal volumes of activated partial thromboplastin reagent, Factor IX deficient plasma isolated from a patient with hemophilia B using sterile phlebotomy techniques well known in the art, and normal pooled plasma as standard, or the sample. In this assay, one unit of activity is defined as that amount present in one milliliter of normal pooled plasma. Further, an assay for biological activity based on the ability of Factor IX to reduce the clotting time of plasma from Factor IX-deficient patients to normal can be performed as described in, for example, Proctor and Rapaport (1961, Amer. J. Clin. Path. 36: 212).

#### E. FSH

The present invention further includes a method for remodeling and/or modifying FSH. Human reproductive function is controlled in part by a family of heterodimeric human glycoprotein hormones which have a common 92 amino acid glycoprotein alpha subunit, but differ in their hormone-specific beta subunits. The family includes follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyrotropin or thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG). Human FSH and LH are used

therapeutically to regulate various aspects of metabolism pertinent to reproduction in the human female. For example, FSH partially purified from urine is used clinically to stimulate follicular maturation in anovulatory women with anovulatory syndrome or luteal phase deficiency. Luteinizing hormone (LH) and FSH are used in combination to stimulate the development of ovarian follicles for *in vitro* fertilization. The role of FSH in the reproductive cycle is sufficiently well-known to permit therapeutic use, but difficulties have been encountered due, in part, to the heterogeneity and impurity of the preparation from native sources. This heterogeneity is due to variations in glycosylation pattern.

FSH is a valuable tool in both *in vitro* fertilization and stimulation of fertilization *in vivo*, but as stated above, its clinical efficacy has been hampered by inconsistency in glycosylation of the protein. It therefore seems apparent that a method for remodeling FSH will be of great benefit to the reproductive sciences.

A remodeled FSH peptide may be administered to a patient selected from the group consisting of a patient undergoing intrauterine insemination (IUI), a patient undergoing *in vitro* fertilization (IVF), and an infertile patient. A remodeled FSH peptide may also be administered to induce or increase ovulation in a patient, to stimulate development of an ovarian follicle in a patient, to induce gametogenic follicle growth in a patient, to stimulate, induce or increase follicle development and subsequent ovulation in a patient, or to treat infertility in a patient. Preferably, the patient is a human female patient. A remodeled FSH peptide may also be administered to a patient having a pituitary deficiency or to a patient during puberty. Preferably this patient is a human male patient.

FSH has been cloned and sequenced, the nucleic and amino acid sequences of which are presented herein as SEQ ID NO:11, SEQ ID NO: 12, respectively (alpha subunit) and SEQ ID NO:13 and SEQ ID NO:14, respectively (beta subunit) (Figure 63A, 63B, 63C and 63D, respectively). The skilled artisan will readily appreciate that the present invention is not limited to the sequences depicted herein, as variants of FSH are well known in the art. As a non-limiting example, U.S. Patent No. 5,639,640 describes the beta subunit comprising two different amino acid sequences and U.S. Patent No. 5,338,835 describes a beta subunit comprising an additional amino acid sequence of approximately twenty-seven amino acids derived from the beta subunit of human chorionic gonadotropin. Therefore, the present



invention comprises FSH variants, both natural and engineered by the human hand, all well known in the art.

Methods to express FSH in cells, both prokaryotic and eukaryotic, are well known in the art and abundantly described in the literature (U.S. Patent Nos. 4,840,896, 4,923,805, 5,156,957). Further, methods for evaluating the biological activity of a remodeled FSH molecule of the present invention are well known in the art, and are described in, for example, U.S. Patent No. 4,589, 402, in which methods for determining the effect of FSH on fertility, egg production, and pregnancy rates is described in both non-human primates and human subjects.

#### F. EPO

The present invention further comprises a method of remodeling and/or modifying EPO. EPO is an acidic glycoprotein of approximately 34 kDa and may occur in three natural forms: alpha, beta, and asialo. The alpha and beta forms differ slightly in carbohydrate components but have the same potency, biological activity and molecular weight. The asialo form is an alpha or beta form with the terminal sialic acid removed. EPO is present in very low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal concentration is enough to stimulate replacement of red blood cells which are lost normally through aging. The amount of erythropoietin in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may be caused by loss of large amounts of blood through hemorrhage, destruction of red blood cells by over-exposure to radiation, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or various forms of anemia. Therefore EPO is a useful compound for replenishing red blood cells after radiation therapy, anemia, and other life-threatening conditions.

A remodeled EPO peptide may be administered to a patient selected from the group consisting of a patient having anemia, an anemic patient having chronic renal insufficiency, an anemic patient having end stage renal disease, an anemic patient undergoing dialysis, an anemic patient having chronic renal failure, an anemic Zidovudine-treated HIV infected patient, an anemic patient having non-myeloid cancer and undergoing chemotherapy, and an

anemic patient scheduled to undergo non-cardiac, non-vascular surgery. A remodeled EPO peptide may also be administered to a patient undergoing surgery to reduce the need for an allogenic blood transfusion. A remodeled EPO peptide may also be administered to a patient at increased risk for a perioperative blood transfusion with significant anticipated blood loss.

5 Preferably, the patient is a human patient.

In light of the importance of EPO in aiding in the recovery from a variety of diseases and disorders, the present invention is useful for the production of EPO with a natural, and therefore more effective saccharide component. EPO, as it is currently synthesized, lacks the full glycosylation complement, and must therefore be administered more frequently and in  
10 higher doses due to its short life in the body. The invention also provides for the production of PEGylated EPO molecules with greatly improved half-life compared with what might be achieved by maximizing desirable glycoforms.

EPO has been cloned and sequenced, and the nucleotide and amino acid sequences are present herein as SEQ ID NO:15 and SEQ ID NO:16, respectively (Figure 64A and 64B,  
15 respectively). It will be readily understood by one of skill in the art that the sequences set forth herein are only an example of the sequences encoding and comprising EPO. As an example, U.S. Patent No. 6,187,564 describes a fusion protein comprising the amino acid sequence of two or more EPO peptides, U.S. Patent Nos. 6,048,971 and 5,614,184 describe mutant EPO molecules having amino acid substitutions at positions 101, 103, 104, and 108.  
20 U.S. Patent No. 5,106,954 describes a truncated EPO molecule, and U.S. Patent No. 5,888,772 describes an EPO analog with substitutions at position 33, 139, and 166. Therefore, the skilled artisan will realize that the present invention encompasses EPO and EPO derivatives and variants as are well documented in the literature and art as a whole.

Additionally, methods of expressing EPO in a cell are well known in the art. As  
25 exemplified in U.S. Patent Nos. 4,703,008, 5,688,679, and 6,376,218, among others, EPO can be expressed in prokaryotic and eukaryotic expression systems. Methods for assaying the biological activity of EPO are equally well known in the art. As an example, the Krystal assay (Krystal, 1983, Exp. Hematol. 11:649-660) can be employed to determine the activity of EPO prepared according to the methods of the present invention. Briefly, the assay  
30 measures the effect of erythropoietin on intact mouse spleen cells. Mice are treated with phenylhydrazine to stimulate production of erythropoietin-responsive red blood cell

progenitor cells. After treatment, the spleens are removed, intact spleen cells are isolated and incubated with various amounts of wild-type erythropoietin or the erythropoietin proteins described herein. After an overnight incubation,  $^3\text{H}$ -thymidine is added and its incorporation into cellular DNA is measured. The amount of  $^3\text{H}$ -thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of erythropoietin with its cellular receptor. The concentration of the erythropoietin protein of the present invention, as well as the concentration of wild-type erythropoietin, is quantified by competitive radioimmunoassay methods well known in the art. Specific activities are calculated as international units measured in the Krystal assay divided by micrograms as measured as immunoprecipitable protein by radioimmunoassay.

Several different mutated EPO's with different glycosylation patterns have been reported. Many have improved stimulation of reticulocytosis activity without effecting the half-life of the peptide in the blood stream of the animal. It is contemplated that mutated EPO peptides can be used in place of the native EPO peptides in any of the glycan remodeling, glycoPEGylation and/or glycoconjugation embodiments described herein. Preferred mutations of EPO are listed in the following table, but not limited to those listed in the table (see, for example, Chern et al., 1991, Eur. J. Biochem. 202:225-229; Grodberg et al., 1993, Eur. J. Biochem. 218:597-601; Burns et al., 2002, Blood 99:4400-4405; U.S. Patent No. 5,614,184; GenBank Accession No. AAN76993; O'Connell et al., 1992, J. Biol. Chem. 267:25010-25018; Elliott et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:2708-2712; Biossel et al., 1993, J. Biol. Chem. 268:15983-15993). The most preferred mutations of EPO are Arg<sup>139</sup> to Ala<sup>139</sup>, Arg<sup>143</sup> to Ala<sup>143</sup> and Lys<sup>154</sup> to Ala<sup>154</sup>. The preferred native EPO from which to make these mutants is the 165 aa form, which is depicted in Fig. 65; however other native forms of EPO may also be used. Finally, the mutations described in Table 10 may be combined with each other and with other mutations to make EPO peptides that are useful in the present invention.

**Table 10. Mutations of EPO.**

Mutation	Citation	Notes
Arg <sup>139</sup> to Ala <sup>139</sup>	J. Biol. Chem. 269:22839 (1994)	Increased activity in bioassays of 120% to 150%.
Arg <sup>143</sup> to Ala <sup>143</sup>		Increased activity in bioassays than native

Lys <sup>154</sup> to Ala <sup>154</sup>	J. Biol. Chem. 269:22839 (1994)	EPO. Increased activity in bioassays of 120% to 150%.
Ser <sup>126</sup> to Met <sup>126</sup>		
Met <sup>54</sup> to Leu <sup>54</sup>	U.S. Patent No. 4,385,260	
Met <sup>54</sup> to Leu <sup>54</sup>	U.S. Patent No. 4,385,260	
Asn <sup>38</sup> to Gln <sup>38</sup>		
Δ1-30	Funakoshi et al., 1993, Biochem. Biophys. Res. Commun. 195:717-722.	Mutant isolated from hepatocellular carcinoma.
Ser <sup>131</sup> Leu <sup>132</sup> to Asn <sup>131</sup> Phe <sup>132</sup>	Genbank Accession No. AAD13964.	
Pro <sup>149</sup> to Gln <sup>149</sup>		
Gly <sup>101</sup> to Ala <sup>101</sup>	U.S. Patent No. 5,615,184 (1994)	Increased activity in bioassays of 120% to 150%.
Ser <sup>147</sup> to Ala <sup>147</sup> and/or Ile <sup>146</sup> to Ala <sup>146</sup>	Wen et al., 1994, J. Biol. Chem. 269:22839-22846.	Mutation results in increased bioactivity.
Ser <sup>126</sup> to Thr <sup>126</sup>	J. Biol. Chem. 267:25010 (1992)	

#### G. GM-CSF

The present invention encompasses a method for the modification of GM-CSF. GM-CSF is well known in the art as a cytokine produced by activated T-cells, macrophages, endothelial cells, and stromal fibroblasts. GM-CSF primarily acts on the bone marrow to increase the production of inflammatory leukocytes, and further functions as an endocrine hormone to initiate the replenishment of neutrophils consumed during inflammatory functions. Further GM-CSF is a macrophage-activating factor and promotes the differentiation of Lagerhans cells into dendritic cells. Like G-CSF, GM-CSF also has clinical applications in bone marrow replacement following chemotherapy.

While G-CSF has demonstrated itself as an important and useful compound for therapeutic applications, present methods for the production of G-CSF from recombinant cells results in a product with a rather short biological life, an inaccurate glycosylation pattern that could potentially lead to immunogenicity, loss of function, an increased need for both larger and more frequent doses in order to achieve the same effect, and the like.

A remodeled GM-CSF peptide may be administered to a patient selected from the group consisting of a patient having Acute Myelogenous Leukemia (AML) or acute non-lymphocytic leukemia (ANLL), a patient undergoing leukapheresis to collect hematopoietic progenitor cells from the peripheral blood, a patient undergoing transplantation of autologous peripheral blood progenitor cells, a non-Hodgkin's lymphoma (NHL) patient undergoing an autologous bone marrow transplant, a Hodgkin's disease patient undergoing an autologous bone marrow transplant, and an acute lymphoblastic leukemia (ALL) patient undergoing an autologous bone marrow transplant. A remodeled GM-CSF peptide may also be administered to a patient to accelerate myeloid engraftment, to shorten time to neutrophil recovery following chemotherapy, to mobilize hematopoietic progenitor cells into the peripheral blood for collection by leukapheresis, or to promote myeloid reconstitution after autologous or allogeneic bone marrow transplantation (BMT). A remodeled GM-CSF peptide may also be administered to a patient in which bone marrow transplantation has failed or in which myeloid engraftment is delayed. Preferably, the patient is a human patient.

GM-CSF has been isolated and cloned, the nucleic acid and amino acid sequences of which are presented as SEQ ID NO:17 and SEQ ID NO:18, respectively (Figure 66A and 66B, respectively). The present invention encompasses a method for modifying GM-CSF, particularly as it relates to the ability of GM-CSF to function as a potent and functional biological molecule. The skilled artisan, when equipped with the present disclosure and the teachings herein, will readily understand that the present invention provides compositions and methods for the modification of GM-CSF.

The present invention further encompasses GM-CSF variants, as well known in the art. As an example, but in no way meant to be limiting to the present invention, a GM-CSF variant has been described in WO 86/06358, where the protein is modified for an alternative quaternary structure. Further, U.S. Patent No. 6,287,557 describes a GM-CSF nucleic acid sequence ligated into the genome of a herpesvirus for gene therapy applications.

Additionally, European Patent Publication No. 0288809 (corresponding to PCT Patent Publication No. WO 87/02060) reports a fusion protein comprising IL-2 and GM-CSF. The IL-2 sequence can be at either the N- or C-terminal end of the GM-CSF such that after acid cleavage of the fusion protein, GM-CSF having either N- or C-terminal sequence

modifications can be generated. Therefore, GM-CSF derivatives, mutants, and variants are well known in the art, and are encompassed within the methods of the present invention.

The expression and activity of a modified GM-CSF molecule of the present invention can be assayed using methods well known in the art, and as described in, for example, U.S.

5 Patent No. 4,810,643. As an example, activity can be measured using radio-labeled thymidine uptake assays. Briefly, human bone marrow from healthy donors is subjected to a density cut with Ficoll-Hypaque (1.077 g/ml, Pharmacia, Piscataway, NJ) and low density cells are suspended in Iscove's medium (GIBCO, La Jolla, CA) containing 10% fetal bovine serum, glutamine and antibiotics. About  $2 \times 10^4$  human bone marrow cells are incubated  
10 with either control medium or the GM-CSF or the present invention in 96-well flat bottom plates at about 37° C in 5% CO<sub>2</sub> in air for about 2 days. Cultures are then pulsed for about 4 hours with 0.5 µCi/well of <sup>3</sup>H-thymidine (New England Nuclear, Boston, Mass.) and uptake is measured as described in, for example, Ventua, et al.(1983, Blood 61:781). An increase in <sup>3</sup>H-thymidine incorporation into human bone marrow cells as compared to bone marrow cells  
15 treated with a control compound is an indication of a active and viable GM-CSF compound.

#### H. IFN-gamma

It is an object of the present invention to encompass a method of modifying and/or remodeling IFN-gamma. IFN-gamma, otherwise known as Type II interferon, in contrast to  
20 IFN alpha and IFN beta, is a homodimeric glycoprotein comprising two subunits of about 21-24 kDa. The size variation is due to variable glycosylation patterns, usually not replicated when reproduced recombinantly in various expression systems known in the art. IFN-gamma is a potent activator of macrophages, increases MHC class I molecule expression, and to a lesser extent, a MHC class II molecule stimulatory agent. Further, IFN-gamma promotes T-  
25 cell differentiation and isotype switching in B-cells. IFN-gamma is also well documented as a stimulator of neutrophils, NK cells, and antibody responses leading to phagocyte-mediated clearance. IFN-gamma has been proposed as a treatment to be used in conjunction with infection by intracellular pathogens, such as tuberculosis and leishmania, and also as an anti-proliferative therapeutic, useful in conditions with abnormal cell proliferation as a hallmark,  
30 such as various cancers and other neoplasias.

IFN-gamma has demonstrated potent immunological activity, but due to variations in glycosylation from systems currently used to express IFN-gamma, the potency, efficacy, biological half-life, and other important factors of a therapeutic have been variable at best. The present invention encompasses methods to correct this crucial defect.

5 A remodeled interferon-gamma peptide may be administered to a patient selected from the group consisting of a patient having chronic granulomatous disease, a patient having malignant osteopetrosis, a patient having pulmonary fibrosis, a patient having tuberculosis, a patient having *Cryptococcal* meningitis, and a patient having pulmonary *Mycobacterium avium* complex (MAC) infection. Preferably, the patient is a human patient.

10 The nucleotide and amino acid sequences of IFN-gamma are presented herein as SEQ ID NO:19 and SEQ ID NO:20, respectively (Figure 67A and 67B, respectively). It will be readily understood that the sequences set forth herein are in no way limiting to the present invention. In contrast, variants, derivatives, and mutants of IFN-gamma are well known to the skilled artisan. As an example, U.S. Patent No. 6,083,724 describes a recombinant avian  
15 IFN-gamma and U.S. Patent No. 5,770,191 describes C-terminus variants of human IFN-gamma. In addition, U.S. Patent No. 4,758,656 describes novel IFN-gamma derivatives, and methods of synthesizing them in various expression systems. Therefore, the present invention is not limited to the sequences of IFN-gamma disclosed elsewhere herein, but encompasses all derivatives, variants, muteins, and the like well known in the art.

20 Expression systems for IFN-gamma are equally well known in the art, and include prokaryotic and eukaryotic systems, as well as plant and insect cell preparations, methods of which are known to the skilled artisan. As an example, U.S. Patent No. 4,758,656 describes a system for expressing IFN-gamma derivatives in *E. coli*, whereas U.S. Patent No. 4,889,803 describes an expression system employing Chinese hamster ovary cells and an SV40  
25 promoter.

Assays for the biological activity of a remodeled IFN-gamma prepared according to the methods disclosed herein will be well known to one of skill in the art. Biological assays for IFN-gamma expression can be found in, for example, U.S. Patent No. 5,807,744. Briefly, IFN-gamma is added to cultures of CD34<sup>++</sup>CD38<sup>-</sup> cells (100 cells per well) stimulated by  
30 cytokine combinations to induce proliferation of CD34<sup>++</sup>CD38<sup>-</sup> cells, such as IL-3, c-kit ligand and either IL-1, IL-6 or G-CSF. Cell proliferation, and generation of secondary

colony forming cells will be profoundly inhibited in a dose dependent way, with near complete inhibition occurring at 5000 U/milliliter of IFN-gamma. As a confirmatory test to the inhibitory effect of IFN-gamma, addition of IFN-gamma antibodies can be performed as a control.

#### I. alpha-Protease inhibitor ( $\alpha$ -antitrypsin)

The present invention further includes a method for the remodeling of alpha-protease inhibitor (A-1-PI,  $\alpha$ -1-antitrypsin or  $\alpha$ -1-trypsin inhibitor), also known as alpha-antitrypsin. A-1-PI is a glycoprotein having molecular weight of 53 kDa. A-1-PI plays a role in  
0 controlling tissue destruction by endogenous serine proteases, and is the most pronounced serine protease inhibitor in blood plasma. In particular, A-1-PI inhibits various elastases including neutrophil elastase. Elastase is a protease which breaks down tissues, and can be particularly problematic when its activity is unregulated in lung tissue. This protease  
functions by breaking down foreign proteins. However, when API is not present in sufficient  
15 quantities to regulate elastase activity, the elastase breaks down lung tissue. In time, this imbalance results in chronic lung tissue damage and emphysema. In fact, a genetic deficiency of A-1-PI has been shown to be associated with premature development of pulmonary emphysema. A-1-PI replenishment has been successfully used for treatment of this form of emphysema. Further, a deficiency of A-1-PI may also contribute to the  
20 aggravation of other diseases such as cystic fibrosis and arthritis, where leukocytes move in to the lungs or joints to fight infection.

Therefore, A-1-PI could conceivably be used to treat diseases where an imbalance between inhibitor and protease(s), especially neutrophil elastase, is causing progression of a disease state. Antiviral activity has also been attributed to A-1-PI. In light of this, it logically  
25 follows that the present invention is useful for the production of A-1-PI that is safe, effective, and potent in the ever changing atmosphere of the lungs.

A remodeled A-1-PI peptide may be administered to a patient selected from the group consisting of a patient having congenital alpha-1-antitrypsin deficiency and emphysema, a patient having cystic fibrosis, and a patient having pulmonary fibrosis. Preferably, the patient  
30 is a human patient.



A-1-PI has been cloned and sequenced, and is set forth in SEQ ID NO:21 and SEQ ID NO:22 (Figure 68A and 68B, respectively). As is understood by one of skill in the art, natural and engineered variants of A-1-PI exist, and are encompassed in the present invention. As an example, U.S. Patent No. 5,723,316 describes A-1-PI derivatives having amino acid substitutions at positions 356-361 and further comprises an N-terminal extension of approximately three amino acids. U. S. Patent No. 5,674,708 describes A-1-PI analogs with amino acid substitutions at position 358 in the primary amino acid sequence. The skilled artisan will readily realize that the present invention encompasses A-1-PI variants, derivatives, and mutants known or to be discovered.

Methods for the expression and determination of activity of a remodeled A-1-PI produced according to the methods of the present invention are well known in the art, and are described in, for example, U.S. Patent No. 5,674,708 and U.S. Patent No. 5,723,316. Briefly, biological activity can be determined using assays for antichymotrypsin activity by measuring the inhibition of the chymotrypsin-catalyzed hydrolysis of substrate N-suc-Ala--Ala--Pro--Phe-p-nitroanilide (0.1 ml of a 10 mM solution in 90% DMSO), as described in, for example, DelMar et al. (1979, Anal. Biochem. 99: 316). A typical chymotrypsin assay contains, in 1.0 milliliters: 100 mM Tris-Cl buffer, pH 8.3, 0.005% (v/v) Triton X-100, bovine pancreatic chymotrypsin (18 kmmol) and A-1-PI of the present invention. The assay mixture is pre-incubated at room temperature for 5 minutes, substrate (0.01 ml of a 10 mM solution in 90% DMSO) is added and remaining chymotrypsin activity is determined by the rate of change in absorbance at 410nm caused by the release of p-nitroaniline. Measurements of optical absorbance are conducted at 25° C using a spectrophotometer fitted with a temperature controlled sample compartment.

#### 25        J. Glucocerebrosidase

The invention described herein further includes a method for the modification of glucocerebrosidase. Glucocerebrosidase is a lysosomal glycoprotein enzyme which catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide. Variants of glucocerebrosidase are sold commercially as Cerezyme™ and Ceredase™, and is an approved therapeutic for the treatment of Gaucher disease. Ceredase™ is a placental derived form of glucocerebrosidase with complete N-linked structures. Cerezyme™ is a recombinant

variant of glucocerebrosidase which is 497 amino acids in length and is expressed in CHO cells. The 4 N-linked glycans of Cerezyme have been modified to terminate in the trimannose core.

Glucocerebrosidase is presently produced in recombinant mammalian cell cultures, and therefore reflects the glycosylation patterns of those cells, usually rodent cells such as Chinese hamster ovary cells or baby hamster kidney cells, which differ drastically from those of human glycosylation patterns, leading to, among other things, immunogenicity and lack of potency.

A remodeled glucocerebrosidase peptide may be administered to a patient selected from the group consisting of a patient having a lysosomal storage disease, a patient having a glucocerebrosidase deficiency, and a patient having Gaucher disease. Preferably, the patient is a human patient.

The nucleic acid and amino acid sequences of glucocerebrosidase are set forth herein as SEQ ID NO:23 and 24 (Figure 69A and 69B, respectively). However, as will be appreciated by the skilled artisan, the sequences represented herein are prototypical sequences, and do not limit the invention. In fact, variants of glucocerebrosidase are well known, and are described in, for example, U.S. Patent 6,015,703 describes enhanced production of glucocerebrosidase analogs and variants thereof. Further, U.S. Patent No. 6,087,131 describes the cloning and sequencing of yet another glucocerebrosidase variant. It is the intention of the present invention to encompass these and other derivatives, variants, and mutants known or to be discovered in the future.

Methods for the expression of glucocerebrosidase are well known in the art using standard techniques, and are described in detail in, for example, U.S. Patent No. 6,015,703. Assays for the biological efficacy of a glucocerebrosidase molecule prepared according to the methods of the present invention are similarly well known in the art, and a mouse Gaucher disease model for evaluation and use of a glucocerebrosidase therapeutic is described in, for example, Marshall et al. (2002, Mol. Ther. 6:179).

#### K. TPA

The present invention further encompasses a method for the remodeling of tissue-type activator (TPA). TPA activates plasminogen to form plasmin which dissolves fibrin, the

main component of the protein substrate of the thrombus. TPA preparations were developed as a thrombolytic agents having a very high selectivity toward the thrombus in the thrombolytic treatment for thrombosis which causes myocardial infarction and cerebral infarction.

5 Further, various modified TPA's have been produced by genetic engineering for the purpose of obtaining higher affinity to fibrin and longer half-life in blood than that of natural TPA. TPA's are proteins that are generally extremely difficult to solubilize in water. In particular, the modified TPA's are more difficult to solubilize in water than natural TPA, making very difficult the preparation of modified TPA's. Modified TPA's are thus difficult to  
10 dissolve in water at the time of the administration to a patient. However, the modified TPA's have various advantages, such as increased affinity for fibrin and longer half-life in blood. It is the object of the present invention to increase the solubility of modified TPA's.

A remodeled TPA peptide may be administered to a patient selected from the group consisting of a patient suffering from an acute myocardial infarction and a patient suffering  
15 from an acute ischemic stroke. A remodeled TPA peptide may also be administered to a patient to improve ventricular function following an acute myocardial infarction, to reduce the incidence of congestive heart failure following an acute myocardial infarction, or to reduce mortality associated with acute myocardial infarction. A remodeled TPA peptide may also be administered to a patient to improve neurological recovery following an acute  
20 ischemic stroke or to reduce the incidence of disability or paralysis following an acute ischemic stroke. Preferably, the patient is a human patient.

The nucleic and amino acid sequences of TPA are set forth herein as SEQ ID NO:25 and SEQ ID NO:26, respectively (Figure 70A and 70B, respectively). As described above, variants of TPA have been constructed and used in therapeutic applications. For example,  
25 U.S. Patent 5,770,425 described TPA variants in which some of all of the fibrin domain has been deleted. Further, U.S. Patent 5,736,134 describes TPA in which modifications to the amino acid at position 276 are disclosed. The skilled artisan, when equipped with the present disclosure and the teachings herein, will readily realize that the present invention comprises the TPA sequences set forth herein, as well as those variants well known to one versed in the  
30 literature.

The expression of TPA from a nucleic acid sequence encoding the same is well known in the art, and is described, in detail, in, for example, U.S. Patent No. 5,753,486. Assays for determining the biological properties of a TPA molecule prepared according to the methods of the present invention are similarly well known in the art. Briefly, a TPA molecule synthesized as disclosed elsewhere herein can be assayed for their ability to lyse fibrin in the presence of saturating concentrations of plasminogen, according to the method of Carlsen et al. (1988, Anal. Biochem. 168: 428 ). The *in vitro* clot lysis assay measures the activity of tissue-type activators by turbidimetry using a microcentrifugal analyzer. A mixture of thrombin and TPA is centrifuged into a mixture of fibrinogen and plasminogen to initiate clot formation and subsequent clot dissolution. The resultant profile of absorbance versus time is analyzed to determine the assay endpoint. Activities of the TPA variants are compared to a standard curve of TPA. The buffer used throughout the assay is 0.06M sodium phosphate, pH 7.4 containing 0.01% (v/v) TWEEN 80 and 0.01% (w/v) sodium azide. Human thrombin is at a concentration of about 33 units/ml. Fibrinogen (at 2.0 mg/ml clottable protein) is chilled on wet ice to precipitate fibronectin and then gravity filtered. Glu-plasminogen is at a concentration of 1 mg/ml. The analyzer chamber temperature is set at 37° C. The loader is set to dispense 20 microliters of TPA (about 500 nanograms/milliliter to about 1.5 micrograms per milliliter) as the sample for the standard curve, or 20 microliters of variant TPAs at a concentration to cause lysis within the range of the standard curve. Twenty microliters of thrombin as the secondary reagent, and 200 microliters of a 50:1 (v/v) fibrinogen: plasminogen mixture as the primary reagent. The absorbance/time program is used with a 5 min incubation time, 340-nanometer-filter and 90 second interval readings.

#### L. IL-2

The present invention further encompasses a method for the remodeling and modification of IL-2. IL-2 is the main growth factor of T lymphocytes and increases the humoral and cellular immune responses by stimulating cytotoxic CD8 T cells and NK cells. IL-2 is therefore crucial in the defense mechanisms against tumors and viral infections. IL-2 is also used in therapy against metastatic melanoma and renal adenocarcinoma, and has been used in clinical trials in many forms of cancer. Further, IL-2 has also been used in HIV infected patients where it leads to a significant increase in CD4 counts.

Given the success IL-2 has demonstrated in the management and treatment of life-threatening diseases such as various cancers and AIDS, it follows that the methods of the present invention would be useful for developing an IL-2 molecule that has a longer biological half-life, increased potency, and in general, a therapeutic profile more similar to wild-type IL-2 as it is synthesized and secreted in the healthy human.

A remodeled IL-2 peptide may be administered to a patient selected from the group consisting of a patient having metastatic renal cell carcinoma, a patient having metastatic melanoma, a patient having ovarian cancer, a patient having Acute Myelogenous Leukemia (AML), a patient having non-Hodgkin's lymphoma (NHL), a patient infected with HIV, and a patient infected with Hepatitis C. A remodeled IL-2 peptide may also be useful for administration to a patient as a cancer vaccine adjuvant. Preferably, the patient is a human patient.

IL-2 has been cloned and sequenced, and the nucleic acid and amino acid sequences are presented herein as SEQ ID NO:27 and SEQ ID NO:28 (Figure 71A and 71B, respectively). The present invention should in no way be construed as limited to the IL-2 nucleic acid and amino acid sequences set forth herein. Variants of IL-2 are described in, for example, U.S. Patent No. 6,348,193, in which the asparagine at position 88 is substituted for arginine, and in U.S. Patent No. 5,206,344, in which a polymer comprising IL-2 variants with various amino acid substitutions is described. The present invention encompasses these IL-2 variants and others well known in the art.

Methods for the expression and to determine the activity of IL-2 are well known in the art, and are described in, for example, U.S. Patent No. 5,417,970. Briefly, expression of IL-2, or variants thereof, can be accomplished in a variety of both prokaryotic and eukaryotic systems, including *E. coli*, CHO cells, BHK cells, insect cells using a baculovirus expression system, all of which are well known in the art.

Assays for the activity of a modified IL-2 prepared according to the methods of the present invention can proceed as follows. Peripheral blood lymphocytes can be separated from the erythrocytes and granulocytes by centrifuging on a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient by the method described in, for example, A. Boyum et al. (Methods in Enzymology, 1984, Vol. 108, page 88, Academic Press, Inc.). Lymphocytes are subsequently washed about three times in culture medium consisted of RPMI 1640 (Gibco-

BRL, La Jolla, CA) plus 10% AB human serum (CTS Purpan, Toulouse, France) inactivated by heat (1 hour at 56° C), 2 mM sodium pyruvate, 5 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (complete medium). Adhesive cells (monocytes and macrophages) are eliminated by adhesion to plastic and the remainder of the cells are suspended in complete medium at a concentration of about 5 to 10 X10<sup>5</sup> cells per milliliter and seeded in culture flasks at a density of about 1-2 X 10<sup>5</sup> cells per square centimeter. Flasks are then incubated at 37° C in a 5% CO<sub>2</sub> atmosphere for about 1 hour, after which the non-adhesive lymphocytes are recovered by aspiration after gentle agitation of the culture flasks.

Non-adhesive lymphocytes are washed once and cultivated at a concentration of about 10<sup>5</sup> cells per milliliter in complete medium in the presence of the IL-2 of the present invention for about 48 hours in an incubator as described above. The cells are then washed once.

The cytotoxic activity of the cells is evaluated after about 4 hours of contact with target cells of the human T lymphoid line C8166-45/C63 (HT1 cells) resistant to NK cell cytotoxicity, as described by Salahuddin et al. (1983, Virology 129: 51-64; 1984, Science: 223, 703-707). 6 X 10<sup>5</sup> HT1 cells are radio-tagged with about 200 µCi of <sup>51</sup>Cr (sodium chromate, Amersham, Arlington Heights, IL) at 37° C for about 1 hour in complete medium without serum, and then washed several times. The target cells and effective cells are distributed in round-bottomed microtitration plates with varying ratios of effective cells to target cells (50:1, 10:1, 1:1). The microtitration plates are centrifuged and, after incubation as described above, the supernatant from each well is recovered and the radioactivity is measured using a gamma counter. Cytotoxicity is determined from the quantity of <sup>51</sup>Cr released by dead target cells. Non-specific cytotoxicity is determined from the amount of radioactivity spontaneously released from the target cells in the absence of effective cells.

The present method is just one of many well known in the art for measuring the cytotoxicity of effector cells, and is should not be construed as limiting to the present invention.

### M. Factor VIII

The invention further encompasses a method for the remodeling and modification of Factor VIII. As described earlier for Factor VII and Factor IX, Factor VIII is a critical component of the blood coagulation pathway. Human Factor VIII, (antihemophilic factor; FVIII:C) is a human plasma protein consisting of 2 peptides (light chain molecular weight of 80 kDa and heavy chain molecular weight variable from 90 to 220 kDa, depending on glycosylation state). It is an essential cofactor in the coagulation pathway and is required for the conversion of Factor X into its active form (Factor Xa). Factor VIII circulates in plasma as a non-covalent complex with von Willibrand Factor (aka FVIII:RP), a dimer of a 2050 aa peptide (See, U.S. Patent No. 6,307,032). Blood concentrations of Factor VIII below 20% of normal cause a bleeding disorder designated hemophilia A. Factor VIII blood levels less than 1% result in a severe bleeding disorder, with spontaneous joint bleeding being the most common symptom.

Similar to other blood coagulation factors, Factor VIII is a therapeutic with a great deal of potential for the treatment of various bleeding disorders, such as hemophilia A and hemophilia B. Due to the glycosylation of the heavy chain, current methods for the preparation of Factor VIII from recombinant cells results in a product that is not as effective as natural Factor VIII. Purification methods from human plasma result in a crude composition that is less effective and more difficult to prepare than recombinant Factor VIII. The current invention seeks to improve this situation.

A remodeled Factor VIII peptide may be administered to a patient selected from the group consisting of a patient having von Willebrand's disease, a patient having Hemophilia A, a patient having Factor VIII:C deficiency, a patient having fibrinogen deficiency, a patient having Factor XIII deficiency, and a patient having acquired Factor VIII inhibitors (acquired hemophilia). A remodeled Factor VIII peptide may also be administered to a patient to prevent, treat or control bleeding or hemorrhagic episodes. Preferably, the patient is a human patient.

The nucleic acid and amino acid sequences of Factor VIII are presented herein as SEQ ID NO:29 and SEQ ID NO:30, respectively (Figure 72A and 72B, respectively). The art is rife with variants of Factor VIII, as described in, for example, U.S. Patent No. 5,668,108, in which the aspartic acid at position 1241 is replaced by a glutamic acid with the

accompanying nucleic acid changes as well. U.S. Patent No. 5,149,637 describes a Factor VIII variants comprising the C-terminal fraction, either glycosylated or unglycosylated, and U.S. Patent No. 5,661,008 describes a Factor VIII variant comprising amino acids 1-740 linked to amino acids 1649 to 2332 by at least 3 amino acid residues. Therefore, variants,  
5 derivatives, modifications and complexes of Factor VIII are well known in the art, and are encompassed in the present invention.

Expression systems for the production of Factor VIII are well known in the art, and include prokaryotic and eukaryotic cells, as exemplified in U.S. Patent Nos. 5,633,150, 5,804,420, and 5,422,250.

10 To determine the biological activity of a Factor VIII molecule synthesized according to the methods of the present invention, the skilled artisan will recognize that the assays described herein for the evaluation of Factor VII and Factor IX are applicable to Factor VIII.

#### N. Urokinase

15 The present invention also includes a method for the remodeling and/or modification of urokinase. Urokinase is a serine protease which activates plasminogen to plasmin. The protein is synthesized in a variety of tissues including endothelium and kidney, and is excreted in trace amounts into urine. Purified urokinase exists in two active forms, a high molecular weight form (HUK; approximately 50 kDa) and a low molecular weight form  
20 (LUK; approximately 30 kDa). LUK has been shown to be derived from HUK by a proteolysis after lysine 135, releasing the first 135 amino acids from HUK. Conventional wisdom has held that HUK or LUK must be converted to proteolytically active forms by the proteolytic hydrolysis of a single chain precursor, also termed prourokinase, between lysine 158 and isoleucine 159 to generate a two-chain activated form (which continues to  
25 correspond to either HUK or LUK). The proteolytically active urokinase species resulting from this hydrolytic clip contains two amino acid chains held together by a single disulfide bond. The two chains formed by the activation clip are termed the A or A<sub>1</sub> chains (HUK or LUK, respectively), and the B chain comprising the protease domain of the molecule.

Urokinase has been shown to be an effective thrombolytic agent. However, since it is  
30 produced naturally in trace quantities the cost of the enzyme is high for an effective dosage. Urokinase has been produced in recombinant cell culture, and DNA encoding urokinase is



known together with suitable vectors and host microorganisms. Present compositions comprising urokinase and methods for producing urokinase recombinantly are hampered by a product that has deficient glycosylation patterns, and given the complex proteolytic cleavage events surrounding the activation of urokinase, this aberrant glycosylation leads to a less effective and less potent product.

A remodeled urokinase peptide may be administered to a patient selected from the group consisting of a patient having an embolism, a patient having an acute massive pulmonary embolism, and a patient having coronary artery thrombosis. Preferably, the patient is a human patient. A remodeled urokinase peptide may also be used to restore patency to an intravenous catheter, including a central venous catheter obstructed by clotted blood or fibrin.

The sequence of the nucleotides encoding the primary amino acid chain of urokinase are depicted in SEQ ID NO:33 and SEQ ID NO:34 (Figure 73A and 73B, respectively). Variants of urokinase are well known in the art, and therefore the present invention is not limited to the sequences set forth herein. In fact, the skilled artisan will readily realize that urokinase variants described in, for example U.S. Patent Nos. 5,219,569, 5,648,253, and 4,892,826, exist as functional moieties, and are therefore encompassed in the present invention.

The expression and evaluation of a urokinase molecule prepared according to the methods of the present invention are similarly well known in the art. As a non-limiting example, the expression of urokinase in various systems is detailed in U.S. Patent No. 5,219,569. An assay for determining the activity and functionality of a urokinase prepared in accordance to the methods set forth herein are described throughout the literature, and are similar to assays for other plasminogen and fibrin related assays described elsewhere throughout. One example of an assay to determine the activity of an urokinase molecule synthesized as described herein can be as described in, for example, Ploug, et al. (1957, Biochim. Biophys. Acta 24: 278-282), using fibrin plates comprising 1.25% agarose, 4.1 mg/ml human fibrinogen, 0.3 units/ml of thrombin and 0.5 µg/ml of soybean trypsin inhibitor.

### O. Human DNase

The present invention further encompasses a method for the remodeling and/or modification of recombinant human DNase. Human DNase I has been tested as a therapeutic agent and was shown to diminish the viscosity of cystic fibrosis mucus *in vitro*. It has been  
5 determined that purulent mucus contains about 10-13 mg/ml of DNA, an ionic polymer predicted to affect the rheologic properties of airway fluids. Accordingly, bovine pancreatic DNase I, an enzyme that degrades DNA, was tested as a mucolytic agent many years ago but did not enter clinical practice, because of side effects induced by antigenicity and/or contaminating proteases. Recombinant human DNase is currently used as a therapeutic agent  
10 to alleviate the symptoms of diseases such as cystic fibrosis.

A remodeled rDNase peptide may be administered to a patient having cystic fibrosis. A remodeled rDNase peptide may also be administered to a cystic fibrosis patient to improve pulmonary function. Preferably, the patient is a human patient.

Similar to DNase derived from bovine sources, recombinant human DNase poses  
15 some problems, mostly due to lowered efficacy due to improper glycosylation imparted by mammalian expression systems currently in use. The present invention describes a method for remodeling DNase, leading to increased efficacy and better therapeutic results.

The nucleotide and amino acid sequences of human DNase are presented herein as SEQ ID NO:39 and SEQ ID NO:40 (Figure 74A and 74B, respectively). Variants of the  
20 peptide comprising DNase are well known in the art. As an example, U.S. Patent No. 6,348,343 describes a human DNase with multiple amino acid substitutions throughout the primary structure. Additionally, U.S. Patent No. 6,391,607 describes a hyperactive variant of DNase with multiple amino acid substitutions at positions 9, 14, 74, 75, and 205. The present examples, and others well known in the art or to be discovered in the future are encompassed  
25 in the present invention.

Expression systems for producing a DNase peptide are well known to the skilled artisan, and have been described in prokaryotic and eukaryotic systems. For example, PCT Patent Publication No. WO 90/07572 describes these methods in considerable detail.

Assays to determine the biological activity of a DNase molecule developed according  
30 to the methods of the present invention are well known in the art. As an example, but in no way meant to be limiting to the present invention, an assay to determine the DNA-hydrolytic

activity of human DNase I is presented herein. Briefly, two different plasmid digestion assays are used. The first assay ("supercoiled DNA digestion assay") measures the conversion of supercoiled double-stranded plasmid DNA to relaxed (nicked), linear, and degraded forms. The second assay ("linear DNA digestion assay") measured the conversion of linear double-stranded plasmid DNA to degraded forms. Specifically, DNase prepared according to the methods of the present invention is added to 160 microliters of a solution comprising 25 micrograms per milliliter of either supercoiled plasmid DNA or EcoRI-digested linearized plasmid DNA in 25 mM HEPES, pH 7.1, 100 µg/ml bovine serum albumin, 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 150 mM NaCl, and the samples are incubated at room temperature. At various times, aliquots of the reaction mixtures are removed and quenched by the addition of 25 mM EDTA, together with xylene cyanol, bromophenol blue, and glycerol. The integrity of the plasmid DNA in the quenched samples is analyzed by electrophoresis of the samples on agarose gels. After electrophoresis, the gels are stained with a solution of ethidium bromide and the DNA in the gels is visualized by ultraviolet light. The relative amounts of supercoiled, relaxed, and linear forms of plasmid DNA are determined by scanning the gels with a fluorescent imager (such as the Molecular Dynamics Model 575 FluorImager) and quantitating the amount of DNA in the bands of the gel that correspond to the different forms.

#### P. Insulin

The invention further includes a method for remodeling insulin. Insulin is well known as the most effective treatment for type I diabetes, in which the beta islet cells of the pancreas do not produce insulin for the regulation of blood glucose levels. The ramifications of diabetes and uncontrolled blood glucose include circulatory and foot problems, and blindness, not to mention a variety of other complications that either result from or are exacerbated by diabetes.

Prior to the cloning and sequencing of human insulin, porcine insulin was used as a treatment for diabetes. Insulin is now produced recombinantly, but the short, 51 amino acid sequence of the mature molecule is a complex structure comprising multiple sulfide bonds.

Current methods to recombinantly produce insulin result in a product that lacks similarity to

the native protein as produced in healthy non-diabetic subjects. The present invention seeks to repair this flaw.

A remodeled insulin peptide may be administered to a patient selected from the group consisting of a patient having Type I Diabetes (diabetes mellitus) and a patient having Type 2 diabetes mellitus who requires basal (long-acting) insulin for the control of hyperglycemia. A remodeled insulin peptide may also be administered to a diabetic patient to control hyperglycemia. Preferably, the patient is a human patient.

The nucleotide and amino acid sequence of human insulin is portrayed in SEQ ID NO:43 and SEQ ID NO:44, respectively (Figure 75A and 75B, respectively). Variants of insulin are abundant throughout the art. U.S. Patent No. 6,337,194 describes insulin fusion protein analogs, U.S. Patent No. 6,323,311 describes insulin derivatives comprising a cyclic anhydride of a dicarboxylic acid, and U.S. Patent No. 6,251,856 describes an insulin derivative comprising multiple amino acid substitutions and a lipophilic group. The skilled artisan will recognize that the following examples of insulin derivatives are in no way exhaustive, but simply represent a small sample of those well known in the art. Therefore, the present invention comprises insulin derivatives known or to be discovered.

Expression systems for the production of insulin are well known in the art, and can be accomplished using molecular biology techniques as described in, for example, Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York).

Assays to determine the functionality of an insulin molecule prepared according to the methods of the present invention are similarly well known in the art. For example, an *in vivo* model of glucose depression can be used to evaluate the biological activity of insulin synthesized using the methods of the present invention. Useful for this purpose is a rat model. The animals are fasted overnight (16 hours) prior to the experiment, and then anesthetized with intraperitoneally administered sodium pentobarbital or another suitable anesthetic such as ketamine. Each animal receives an i.v. injection (tail vein) of the particular insulin derivative (20 µg/ml/kg). Blood samples are taken from the jugular vein 15 and 5 minutes before injection and 15, 30, 60, 90, 120, 180, and 240 minutes after injection. Blood glucose levels are measured with a blood glucose monitor, available from a variety of commercial suppliers.

Q. Hepatitis B Vaccines (HBsAg)

The present invention further comprises a method for the remodeling the antigen used in hepatitis B vaccines (HbsAg or Hepatitis B sAg). HBsAg is a recombinantly produced surface antigen of the hepatitis B S-protein, and is used to illicit an immune response to the hepatitis B virus, an increasing dangerous virus that results in, among other things, liver disease including cirrhosis and carcinoma, and results in over 1 million deaths worldwide annually. Currently the HBsAg vaccine is administered three times over a six month interval to illicit a protective and neutralizing immune response.

HBsAg is currently produced in yeast strains, and therefore reflects the glycosylation patterns native to a fungus. The present invention provides a method to remodel HBsAg, resulting in among other things, improved immunogenicity, antibodies with improved affinity for the virus, and the like.

A remodeled HBsAg peptide may be administered to a patient to immunize the patient against disease caused by a Hepatitis B virus. A remodeled HBsAg peptide may also be administered to a predialysis patient or a dialysis patient to immunize the patient against disease caused by a Hepatitis B virus. Preferably, the patient is a human patient.

The sequences of the S-protein from a Hepatitis B virus (HBsAg) nucleic acid and primary amino acid chain are set forth herein as SEQ ID NO:45 and SEQ ID NO:46 (Figure 76A and 76B, respectively). The nucleotide is 1203 bases in length. The amino acid is 400 residues long. The last 226 amino acid residues are the small S-antigen, which is used in the GlaxoSmithKline vaccine and the Merck vaccine. Fifty-five amino acids upstream from the small S-antigen is the Pre-S start codon. The Pre-S + S regions are the middle S antigen, which is used in the Aventis Pasteur vaccine. From the first start codon to the Pre-S start codon comprises the rest of the S-protein, and is called the large S-protein. This is but one example of the HBsAg used in vaccines, and other subtypes are well known, as exemplified in GenBank Acc Nos.: AF415222, AF415221, AF415220, and AF415219. The sequences presented herein are simply examples of HBsAg known in the art. Similar antigens have been isolated from other strains of hepatitis B virus, and may or may not have been evaluated for antigenicity and potential as vaccine candidates. The present invention therefore encompasses hepatitis B vaccine S-protein surface antigens known or to be discovered.

Expression of an HBsAg in an expression system is a routine procedure for one of skill in the art, and is described in, for example, U.S. Patent No. 5,851,823. Assays for the immunogenicity of a vaccine are well known in the art, and comprise various tests for the production of neutralizing antibodies, and employ techniques such as ELISA, neutralization assays, Western blots, immunoprecipitation, and the like. Briefly, a sandwich ELISA for the detection of effective anti-HBsAg antibodies is described. The Enzygnost HBsAg assay (Aventis Behring, King of Prussia, PA) is used for such methods. Wells are coated with anti-HBs. Serum plasma or purified protein and appropriate controls are added to the wells and incubated. After washing, peroxidase-labeled antibodies to HBsAg are reacted with the remaining antigenic determinants. The unbound enzyme-linked antibodies are removed by washing and the enzyme activity on the solid phase is determined by methods well known in the art. The enzymatically catalyzed reaction of hydrogen peroxide and chromogen is stopped by adding diluted sulfuric acid. The color intensity is proportional to the HBsAg concentration of the sample and is obtained by photometric comparison of the color intensity of the unknown samples with the color intensities of the accompanying negative and positive control sera.

#### R. Human Growth Hormone

The present invention further encompasses a method for the remodeling of human growth hormone (HGH). The isoform of HGH which is secreted in the human pituitary, consists of 191 amino acids and has a molecular weight of about 21,500. The isoform of HGH which is made in the placenta is a glycosylated form. HGH participates in much of the regulation of normal human growth and development, including linear growth (somatogenesis), lactation, activation of macrophages, and insulin-like and diabetogenic effects, among others.

HGH is a complex hormone, and its effects are varied as a result of interactions with various cellular receptors. While compositions comprising HGH have been used in the clinical setting, especially to treat dwarfism, the efficacy is limited by the absence of glycosylation of the HGH produced recombinantly.

A remodeled HGH peptide may be administered to a patient selected from the group consisting of a patient having a growth hormone deficiency, a patient having Turner

syndrome, a patient having growth failure due to a lack of adequate endogenous growth hormone secretion, a patient having growth failure due to Prader-Willi syndrome (PWS), a patient having growth failure associated with chronic renal insufficiency, and a patient having AIDS associated wasting or cachexia. A remodeled HGH peptide may also be administered  
5 to a patient having short stature. Preferably, the patient is a human patient.

The nucleic and amino acid sequence of HGH are set forth elsewhere herein as SEQ ID NO:47 and SEQ ID NO:48 (Figure 77A and 77B, respectively). The skilled artisan will recognize that variants, derivatives, and mutants of HGH are well known. Examples can be found in U.S. Patent No. 6,143,523 where amino acid residues at positions 10, 14, 18, 21,  
10 167, 171, 174, 176 and 179 are substituted, and in U.S. Patent No. 5,962,411 describes splice variants of HGH. The present invention encompasses these HGH variants known in the art of to be discovered.

Methods for the expression of HGH in recombinant cells is described in, for example, U.S. Patent No. 5,795,745. Methods for expression of HGH in, *inter alia*, prokaryotes,  
15 eukaryotes, insect cell systems, plants, and *in vitro* translation systems are well known in the art.

An HGH molecule produced using the methods of the current invention can be assayed for activity using a variety of methods known to the skilled artisan. For example, U.S. Patent 5,734,024 describes a method to determine the biological functionality of an  
20 expressed HGH.

### S. Anti-Thrombin III

Antithrombin (antithrombin III, AT-III) is a potent inhibitor of the coagulation cascade in blood. It is a non-vitamin K-dependent protease that inhibits the action of  
25 thrombin as well as other procoagulant factors (e.g., Factor Xa). Congenital antithrombin III deficiency is an autosomal dominant disorder in which an individual inherits one copy of a defective gene. This condition leads to increased risk of venous and arterial thrombosis, with onset of clinical manifestations typically presenting in young adulthood. Severe congenital antithrombin III deficiency, in which the individual inherits two defective genes, is an  
30 autosomal recessive condition associated with increased thrombogenesis, typically noted in infancy. Acquired antithrombin III deficiency most commonly is seen in situations where

there is inappropriate activation of the coagulation system. Common conditions that result in acquired antithrombin III deficiency include disseminated intravascular coagulation, microangiopathic hemolytic anemias due to endothelial damage (i.e., Hemolytic-uremic syndrome), and veno-occlusive disease (VOD) seen in patients undergoing bone marrow transplant. AT-III deficiency may be corrected acutely by infusions of AT-III concentrates.

A remodeled AT-III peptide may be administered to a patient selected from the group consisting of a patient having a hereditary AT-III deficiency in connection with a surgical or obstetrical procedure and a hereditary AT-III deficient patient having a thromboembolism. Preferably, the patient is a human patient.

Antithrombin III (AT-III) is an  $\alpha_2$ -glycoprotein of molecular weight 58,000. It is sold commercially as Thrombate III™ (Bayer Corp., West Haven, CT). The nucleic acid and amino acid sequences of human antithrombin III are displayed in Figure 78A (SEQ ID NO:63) and 78B (SEQ ID NO:64), respectively.

Methods to make anti-thrombin III are well known to those in the art. For example, published nucleic acid and amino acid sequences are available for human antithrombin III (see, U.S. Patent No. 4,517,294) and mutants of human antithrombin III (see, U.S. Patent Nos. 5,420,252, 5,618,713, 5,700,663). The methods of the invention may be used with any of these amino acid sequences and any nucleic acid sequences that encode them, but are not limited to these sequences. Exemplary methods to produce recombinant antithrombin III are well known in the art, and several are described in U.S. Patent Nos. 5,420,252, 5,843,705, 6,441,145 and 5,994,628. Exemplary methods to purify recombinant antithrombin III are described in U.S. Patent Nos. 5,989,593, 6,268,487, 6,395,888, 6,395,881, 6,451,978 and 6,518,406.

There are many known uses for recombinant antithrombin III. Antithrombin III can be used as an anticoagulant during surgery (U.S. Patent Nos. 5,252,557, 5,182,259), as part of a pharmaceutical preparation or method to inhibit thrombosis (U.S. Patent Nos. 5,565,471, 6,001,820), and to reduce the adverse side effects of cellular transplantation (U.S. Patent No. 6,387,366). Additionally, antithrombin III preparations can be used to increase placental blood flow (U.S. Patent No. 5,888,964), inhibit fertilization (U.S. Patent No. 5,545,615), treat asthma (U.S. Patent No. 6,355,626) and treat arthritis (U.S. Patent No. 5,252,557) and other inflammatory processes (U.S. Patent No. 6,399,572). Antithrombin III can also be used to



manufacture replacement blood plasma (U.S. Patent Nos. 4,900,720) or prepare a stabilized cellular blood product (U.S. Patent No. 6,139,878) for transfusions. Antithrombin III may be administered as a pharmaceutical preparation (U.S. Patent Nos. 5,084,273, 5,866,122, 6,399,572, 6,156,731 and 6,514,940) or using gene therapy methodology (U.S. Patent No. 6,410,015). Compositions comprising antithrombin III can be used as tissue adhesives (U.S. Patent No. 6,500,427) or lubricants for medical devices that are introduced to the patient (U.S. Patent No. 6,391,832). Antithrombin III can also be used to coat endovascular stents (U.S. Patent Nos. 6,355,055, 6,240,616, 5,985,307, 5,685,847 and 5,222,971), ocular implants (U.S. Patent No. 5,944,753) and prostheses in general (U.S. Patent Nos. 6,503,556, 6,491,965 and 6,451,373). Antithrombin III can also be used in methods to locate an internal bleeding site in a patient (U.S. Patent No. 6,314,314) and to determine hemostatic dysfunction in a patient (U.S. Patent No. 6,429,017).

#### T. Human Chorionic Gonadotropin

Human Chorionic Gonadotropin (hCG) is a glycoprotein composed of an alpha subunit and a beta subunit. HCG is closely related to two other gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), as well as thyroid stimulating hormone (TSH), all three of which are glycoprotein hormones. The alpha subunits of these various glycoprotein hormones are structurally very similar, but the beta subunits differ in amino acid sequence.

The nucleic acid and amino acid sequences of the human chorionic gonadotropin  $\alpha$ -subunit are displayed in Figures 79A (SEQ ID NO:69) and 79B (SEQ ID NO:70), respectively. The nucleic acid and amino acid sequences of the human chorionic gonadotropin  $\beta$ -subunit are displayed in Figures 79C (SEQ ID NO:71) and 79D (SEQ ID NO:72), respectively.

Human chorionic gonadotropin is used in an infertility treatment to promote ovulation or release of an egg from the ovary in women who do not ovulate on their own. Human chorionic gonadotropin is also given to young males to treat undescended or underdeveloped testicles. It is used in men to stimulate the production of testosterone. Some physicians also prescribe human chorionic gonadotropin for men having erectile dysfunction or lack of sexual desire, and for treatment of male "menopause."

A remodeled hCG peptide may be administered to a patient selected from the group consisting of a patient undergoing assisted reproductive technology (ART), a patient undergoing *in vitro* fertilization (IVF), a patient undergoing embryo transfer, an infertile patient, a male patient having prepubertal cryptorchidism not due to anatomical obstruction, and a male patient having hypogonadotropic hypogonadism. A remodeled hCG peptide may also be administered to induce final follicular maturation and early luteinization in an infertile female patient, wherein the infertile female patient has undergone pituitary desensitization and pretreatment with follicle stimulating hormones. A remodeled hCG peptide may also be administered to induce ovulation and pregnancy in an anovulatory infertile patient.

10 Preferably, the patient is a human patient.

Methods to make human chorionic gonadotropin are well known in the art. The heterodimeric hCG can be recombinantly made in any one of many expression systems currently used for industrial manufacture of recombinant proteins. One method of making recombinant hCG is described in U.S. Patent No. 5,639,639. Methods for making recombinant heterodimeric proteins by expressing both subunits in the same cell are, in general, well known in the art, and several methods are described in the U.S. Patent Nos. 5,643,745 (expression in a filamentous fungus), 5,985,611 and 6,087,129 (expression in secretory cells). Alternatively, each subunit can be expressed individually in cells, and the two subunits later brought together *in vitro* for assembly into the heterodimer.

20 Methods for using human chorionic gonadotropin are numerous and well known in the art. Commonly, hCG is used to induce or synchronize ovulation in mammals (see, U.S. Patent Nos. 6,489,288, 5,589,457, 5,532,155, 4,196,123, 4,062,942 and 4,845,077). Additionally, hCG can be used in pregnancy tests, and in particular agglutination-based tests (see, U.S. Patent Nos. 3,991,175, 4,003,988, 4,071,314 and 4,088,749). hCG can also be used in a contraceptive vaccine (see, U.S. Patent Nos. 4,161,519 and 4,966,888). In addition, hCG can be used to treat conditions related to aging and altered hormonal balance such as benign prostatic hypertrophy (see, U.S. Patent No. 5,610,136) and central nervous system diseases common in the elderly (see, U.S. Patent No. 4,791,099).

Alternatively, hCG can be used to detect and treat cancers that express hCG or one of its subunits. hCG-expressing tumors include, but are not limited to, breast, prostate, ovary and stomach carcinomas, and neuroblastomas such as Kaposi's sarcoma. Antibodies can be

raised to hCG which has been glycoremodeled so as to have glycan structures similar to those found on the tumor-expressed hCG, and these antibodies may be used to detect hCG-expressing tumors in patients according to methods well known in the art (see, U.S. Patent Nos. 4,311,688, 4,478,815 and 4,323,546). Additionally, remodeled hCG can be used to  
5 raise an immune response to a tumor that is expressing hCG (see, U.S. Patent Nos. 5,677,275, 5,762,931, 5,877,148, 4,970,071 and 4,966,753).

hCG can also be used in methods to generally immunomodulate an animal, such as described in U.S. Patent Nos. 5,554,595, 5,851,997 and 5,700,781. In addition, hCG can be used as an inhibitor of the matrix metalloprotease in conditions benefiting from such  
10 treatment, such as chronic inflammatory diseases, multiple sclerosis and angiogenesis-dependent diseases (see, U.S. Patent No. 6,444,639).

#### U. $\alpha$ -Iduronidase

$\alpha$ -Iduronidase is sold commercially as Aldurazyme™ (BioMarin and Genzyme). It is  
15 useful for replacement therapy for the treatment of MPS I, a lysosomal storage disease. MPS I (also known as Hurler disease) is a genetic disease caused by the deficiency of alpha-L-iduronidase, an enzyme normally required for the breakdown of certain complex carbohydrates known as glycosaminoglycans (GAGs). The normal breakdown of GAGs is incomplete or blocked if the enzyme is not present in sufficient quantity. The cell is then  
20 unable to excrete the carbohydrate residues and they accumulate in the lysosomes of the cell and cause MPS I.

A remodeled alpha-iduronidase peptide may be administered to a patient selected from the group consisting of a patient having a lysosomal storage disease, a patient having an alpha-L-iduronidase deficiency, a patient having mucopolysaccharidosis I (MPS I), and a  
25 patient having Hurler disease. Preferably, the patient is a human patient.

Methods to produce and purify  $\alpha$ -iduronidase, as well as methods to treat certain genetic disorders including  $\alpha$ -L-iduronidase deficiency and mucopolysaccharidosis I (MPS 1) are described in U.S. Patent No. 6,426,208. The nucleic acid and amino acid sequences of human  $\alpha$ -iduronidase are found in Figures 80A (SEQ ID NO:65) and 80B (SEQ ID NO:66),  
5 respectively.

#### V. $\alpha$ -Galactosidase A

$\alpha$ -Galactosidase A (also known as agalsidase beta) is sold commercially as Fabrazyme™ (Genzyme).  $\alpha$ -Galactosidase A is useful for the treatment of Fabry disease.

10 Fabry disease is a rare, inherited disorder caused by the deficiency of the essential enzyme  $\alpha$ -galactosidase. Without this enzyme, Fabry patients are unable to breakdown a fatty acid substance in their body called globotriasylceramide (GL-3), which accumulates in cells in the blood vessels of the heart, kidney, brain and other vital organs. The progressive buildup of this substance puts patients a risk for stroke, heart attack, kidney damage and debilitating  
15 pain. Most patients develop kidney failure during adulthood, and severe organ complications lead to death around age forty.

A remodeled alpha-galactosidase A peptide may be administered to a patient selected from the group consisting of a patient having a lysosomal storage disease, a patient having an alpha-galactosidase A deficiency, and a patient having Fabry disease. Preferably, the patient  
20 is a human patient.

The  $\alpha$ -galactosidase A enzyme is a lysosomal enzyme which hydrolyzes globotriaosylceramide and related glycolipids which have terminal  $\alpha$ -galactosidase linkages. It is a 45 kDa N-glycosylated protein encoded on the long arm of the X chromosome. The initial glycosylated forms (Mr=55,000 to 58,000) synthesized in human fibroblasts or Chang  
25 liver cells are processed to a mature glycosylated form (Mr=50,000). The mature active enzyme as purified from human tissues and plasma is a homodimer (Bishop et al., 1986, Proc. Natl. Acad. Sci. USA 83: 4859-4863). The nucleic acid and amino acid sequences of  $\alpha$ -galactosidase A are found in Figures 81A (SEQ ID NO:67) and 81B (SEQ ID NO:68). Other useful nucleic acid and amino acid sequences of alpha-galactosidase A are found in  
30 U.S. Patent No. 6,329,191.

References teaching how to make alpha-galactosidase A are found in U.S. Patent Nos. 5,179,023 and 5,658,567 (expression in insect cells), U.S. Patent No. 5,356,804 (expression and secretion from mammalian cells, including CHO cells), U.S. Patent No. 5,401,451 (expression in mammalian cells), U.S. Patent No. 5,580,757 (expression in mammalian cells as a fusion protein) and U.S. Patent No. 5,929,304 (expression in plant cells). Methods for purifying recombinant alpha-galactosidase A are found in U.S. Patent No. 6,395,884.

References teaching how to use alpha-galactosidase A to treat patients include, but are not limited to, U.S. Patent No. 6,066,626 (gene therapy) and U.S. Patent No. 6,461,609 (treatment with the protein). Mutant forms of alpha-galactosidase A that are useful in the methods of the invention include, but are not limited to, those described in U.S. Patent No. 6,210,666.

#### W. Antibodies

The present invention further comprises a method for the remodeling of various antibody preparations including chimeric antibody preparations, including, chimeric TNFR, chimeric anti-glycoprotein IIb/IIIa, chimeric anti-HER2, chimeric anti-RSV, chimeric anti-CD20, and chimeric anti-TNF. Chimeric antibody preparations comprise a human Fc portion from an IgG antibody and the variable regions from a monoclonal antibody specific for an antigen. Other preparations comprise a receptor, for example the 75 kDa TNF receptor, fused to a human IgG Fc portion. These molecules further include Fab fragments comprising light and heavy chains from human and mice. A chimeric TNFR is useful in the treatment of inflammatory diseases, such as rheumatoid arthritis. Chimeric anti-glycoprotein IIb/IIIa is useful in the treatment of cardiac abnormalities, blood clotting, and platelet function disturbances. A chimeric anti-HER2 is useful as a treatment for breast cancer, chimeric anti-RSV is useful for the treatment of respiratory syncytial virus, chimeric anti-CD20 is useful for the treatment of Non-Hodgkin's lymphoma, and chimeric anti-TNF is used for treatment of Crohn's disease.

While these chimeric antibodies have proved useful in the management of varied diseases, administration has to be fairly frequent and at fairly high doses due to the relatively short half-life of a recombinant protein produced in rodent cells. While a majority of the chimeric antibody is human, and therefore regarded as "self" by the immune system, they are

degraded and destroyed due to non-native glycosylation patterns. The present invention proposes to repair this problem, greatly increasing the efficacy of these novel medicines.

#### Antibodies and Methods of their Generation

The term "antibody," as used herein, refers to an immunoglobulin molecule which is  
5 able to specifically bind to a specific epitope on an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and  
10 F(ab)<sub>2</sub>, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

By the term "synthetic antibody" as used herein, is meant an antibody which is  
15 generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody peptide, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using  
20 synthetic DNA or amino acid sequence technology which is available and well known in the art.

Monoclonal antibodies directed against full length or peptide fragments of a peptide or peptide may be prepared using any well known monoclonal antibody preparation procedures, such as those described, for example, in Harlow et al. (1988, In: Antibodies, A  
25 Laboratory Manual, Cold Spring Harbor, NY) and in Tuszynski et al. (1988, Blood, 72:109-115). Quantities of the desired peptide may also be synthesized using chemical synthesis technology. Alternatively, DNA encoding the desired peptide may be cloned and expressed from an appropriate promoter sequence in cells suitable for the generation of large quantities of peptide. Monoclonal antibodies directed against the peptide are generated from mice  
30 immunized with the peptide using standard procedures as referenced herein.

Nucleic acid encoding the monoclonal antibody obtained using the procedures described herein may be cloned and sequenced using technology which is available in the art, and is described, for example, in Wright et al. (1992, Critical Rev. in Immunol. 12(3,4):125-168) and the references cited therein. Further, the antibody of the invention may be

5 "humanized" using the technology described in Wright et al., (*supra*) and in the references cited therein, and in Gu et al. (1997, Thrombosis and Hematocyst 77(4):755-759).

To generate a phage antibody library, a cDNA library is first obtained from mRNA which is isolated from cells, e.g., the hybridoma, which express the desired peptide to be expressed on the phage surface, e.g., the desired antibody. cDNA copies of the mRNA are

10 produced using reverse transcriptase. cDNA which specifies immunoglobulin fragments are obtained by PCR and the resulting DNA is cloned into a suitable bacteriophage vector to generate a bacteriophage DNA library comprising DNA specifying immunoglobulin genes. The procedures for making a bacteriophage library comprising heterologous DNA are well known in the art and are described, for example, in Sambrook and Russell (2001, Molecular

15 Cloning: A Laboratory Manual, Cold Spring Harbor, NY).

Bacteriophage which encode the desired antibody, may be engineered such that the peptide is displayed on the surface thereof in such a manner that it is available for binding to its corresponding binding peptide, e.g., the antigen against which the antibody is directed. Thus, when bacteriophage which express a specific antibody are incubated in the presence of

20 a cell which expresses the corresponding antigen, the bacteriophage will bind to the cell. Bacteriophage which do not express the antibody will not bind to the cell. Such panning techniques are well known in the art and are described for example, in Wright et al., (*supra*).

Processes such as those described above, have been developed for the production of human antibodies using M13 bacteriophage display (Burton et al., 1994, Adv. Immunol. 57:191-280). Essentially, a cDNA library is generated from mRNA obtained from a

25 population of antibody-producing cells. The mRNA encodes rearranged immunoglobulin genes and thus, the cDNA encodes the same. Amplified cDNA is cloned into M13 expression vectors creating a library of phage which express human antibody fragments on their surface. Phage which display the antibody of interest are selected by antigen binding

30 and are propagated in bacteria to produce soluble human immunoglobulin. Thus, in contrast

to conventional monoclonal antibody synthesis, this procedure immortalizes DNA encoding human immunoglobulin rather than cells which express human immunoglobulin.

Remodeling glycans of antibody molecules

The specific glycosylation of one class of peptides, namely immunoglobulins, has a particularly important effect on the biological activity of these peptides. The invention should not be construed to be limited solely to immunoglobulins of the IgG class, but should also be construed to include immunoglobulins of the IgA, IgE and IgM classes of antibodies.

Further, the invention should not be construed to be limited solely to any type of traditional antibody structure. Rather, the invention should be construed to include all types of antibody molecules, including, for example, fragments of antibodies, chimeric antibodies, human antibodies, humanized antibodies, etc.

A typical immunoglobulin molecule comprises an effector portion and an antigen binding portion. For a review of immunoglobulins, see Harlow et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York, and Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY. The effector portion of the immunoglobulin molecule resides in the Fc portion of the molecule and is responsible in part for efficient binding of the immunoglobulin to its cognate cellular receptor. Improper glycosylation of immunoglobulin molecules particularly in the CH2 domain of the Fc portion of the molecule, affects the biological activity of the immunoglobulin.

More specifically with respect to the immunoglobulin IgG, IgG effector function is governed in large part by whether or not the IgG contains an N-acetylglucosamine (GlcNAc) residue attached at the 4-O position of the branched mannose of the trimannosyl core of the N-glycan at Asparagine (Asn) 297 in the CH2 domain of the IgG molecule. This residue is known as a "bisecting GlcNAc." The purpose of adding bisecting GlcNAc to the N-glycan chains of a natural or recombinant IgG molecule or a IgG-Fc-containing chimeric construct is to optimize Fc immune effector function of the Fc portion of the molecule. Such effector functions may include antibody-dependent cellular cytotoxicity (ADCC) and any other biological effects that require efficient binding to FcγR receptors, and binding to the C1 component of complement. The importance of bisecting GlcNAc for achieving maximum



immune effector function of IgG molecules has been described (Lifely et al., 1995, Glycobiology 5 (8): 813-822; Jeffris et al., 1990, Biochem. J. 268 (3): 529-537).

The glycans found at the N-glycosylation site at Asn 297 in the CH2 domain of IgG molecules have been structurally characterized for IgG molecules found circulating in human and animal blood plasma, IgG produced by myeloma cells, hybridoma cells, and a variety of transfected immortalized mammalian and insect cell lines. In all cases the N-glycan is either a high mannose chain or a complete (Man3, GlcNAc4, Gal2, NeuAc2, Fuc1) or variably incomplete biantennary chain with or without bisecting GlcNAc (Raju et al., 2000, Glycobiology 10 (5): 477-486; Jeffris et al., 1998, Immunological. Rev. 163: 59-76; Lerouge et al., 1998, Plant Mol. Biol. 38: 31-48; James et al., 1995, Biotechnology 13: 592-596).

The present invention provides an *in vitro* customized glycosylated immunoglobulin molecule. The immunoglobulin molecule may be any immunoglobulin molecule, including, but not limited to, a monoclonal antibody, a synthetic antibody, a chimeric antibody, a humanized antibody, and the like. Specific methods of generating antibody molecules and their characterization are disclosed elsewhere herein. Preferably, the immunoglobulin is IgG, and more preferably, the IgG is a humanized or human IgG, most preferably, IgG1.

The present invention specifically contemplates using  $\beta$ 1,4-mannosyl-glycopeptide  $\beta$ 1,4-N-acetylglucosaminyltransferase, GnT-III: EC2.4.1.144 as an *in vitro* reagent to glycosidically link N-acetylglucosamine (GlcNAc) onto the 4-O position of the branched mannose of the trimannosyl core of the N-glycan at Asn 297 in the CH2 domain of an IgG molecule. However, as will be appreciated from the disclosure provided herein, the invention should not be construed to solely include the use of this enzyme to provide a bisecting GlcNAc to an immunoglobulin molecule. Rather, it has been discovered that it is possible to modulate the glycosylation pattern of an antibody molecule such that the antibody molecule has enhanced biological activity, i.e., effector function, in addition to potential enhancement of other properties, e.g., stability, and the like.

There is provided in the present invention a general method for removing fucose molecules from the Asn(297) N-linked glycan for the purpose of enhancing binding to Fc-gammaRIIIA, and enhanced antibody-dependent cellular cytotoxicity (see, Shields et al., 2002, J. Biol. Chem. 277:26733-26740). The method entails contacting the antibody molecule with a fucosidase appropriate for the linkage of the fucose molecule(s) on the

antibody glycan(s). Alternately, the recombinant antibody can be expressed in cells that do express fucosyltransferases, such as the Lec13 variant of CHO cells. The removal of fucose from the glycan(s) of the antibody can be done alone, or in conjunction with other methods to remodel the glycans, such as adding a bisecting GlcNAc. Expression of antibodies in cells  
5 lacking GnT-I may also result in Fc glycans lacking core fucose, which can be further modified by the present invention.

There is provided in the present invention a general method for introducing a bisecting GlcNAc for the purpose of enhancing Fc immune effector function in any preparation of IgG molecules containing N-linked oligosaccharides in the CH2 domain,  
10 typically at Asn 297. The method requires that the population of IgG molecules is brought to a state of glycosylation such that the glycan chain is an acceptor for GnT-III. This is accomplished in any one of three ways: 1) by selection or genetic manipulation of a host expression system that secretes IgG with N-glycan chains that are substrates for GnT-III; 2) by treatment of a population of IgG glycoforms with exoglycosidases such that the glycan  
15 structure(s) remaining after exoglycosidase treatment is an acceptor for GnT-III; 3) some combination of host selection and exoglycosidase treatment as in 1) and 2) above plus successive additions of GlcNAc by GnT-I and GnT-II to create an acceptor for GnT-III.

For example, IgG obtained from chicken plasma contains primarily high mannose chains and would require digestion with one or more  $\alpha$ -mannosidases to create a substrate for  
20 addition of GlcNAc to the  $\alpha$ 1,3 mannose branch of the trimannosyl core by GnT-I. This substrate could be the elemental trimannosyl core, Man3GlcNAc2. Treatment of this core structure with a combination of GnT-I, GnT-II, and GnT-III using UDP-GlcNAc as a sugar donor creates Man3GlcNAc5 as shown in Figure 1. The order of action of these  
glycosyltransferases may be varied to optimize the production of the desired product.

25 Optionally, this structure can then be extended by treatment with  $\beta$ 1,4 galactosyltransferase. If required, the galactosylated oligosaccharide can be further extended using  $\alpha$ 2,3- or  $\alpha$ 2,6-sialyltransferase to achieve a completed biantennary structure. Using this method biantennary glycan chains can be remodeled as required for the optimal Fc immune effector function of any therapeutic IgG under development (Figure 3).

30 Alternatively, IgG molecules found in the plasma of most animals or IgG which is secreted as a recombinant product by most animal cells or by transgenic animals typically

include a spectrum of biantennary glycoforms including complete (NeuAc2, Gal2, GlcNAc4, Man3,  $\pm$ Fuc1) (Figure 3) and variably incomplete forms, with or without bisecting GlcNAc (Raju et al., 2000, *Glycobiology* 10 (5): 477-486; Jeffris et al., 1998, *Immunological Rev.* 163: 59-76). To ensure that bisecting GlcNAc is present in the entire population of

5 immunoglobulin molecules so produced, the mixture of molecules can be treated with the following exoglycosidases, successively or in a mixture: neuraminidase,  $\beta$ -galactosidase,  $\beta$ -hexosaminidase,  $\alpha$ -fucosidase. The resulting trimannosyl core can then be remodeled using glycosyltransferases as noted above.

In some cases it may be desired to abolish effector function from existing antibody molecules. The present invention also includes modifying the Fc glycans with appropriate glycosidases and glycosyltransferases to eliminate effector function. Also anticipated is the addition of sugars modified with PEG or other polymers that serve to hinder or abolish binding of Fc receptors or complement to the antibody.

In addition, IgG secreted by transgenic animals or stored as "plantibodies" by transgenic plants have been characterized. An IgG molecule produced in a transgenic plant having N-glycans that contain  $\beta$ 1,2 linked xylose and/or  $\alpha$ 1,3 linked fucose can be treated with exoglycosidases to remove those residues, in addition to the above described exoglycosidases in order to create the trimannosyl core or a Man3GlcNAc4 structure, and are then treated with glycosyltransferases to remodel the N-glycan as described above.

20 The primary novel aspect of the current invention is the application of appropriate glycosyltransferases, with or without prior exoglycosidase treatment, applied in the correct sequence to optimize the effector function of the antibody. In one exemplary embodiment, a bisecting GlcNAc is introduced into the glycans of IgG molecules or other IgG-Fc-chimeric constructs where bisecting GlcNAc is required. In another exemplary embodiment, the core fucose is removed from the glycans of IgG molecules or other IgG-Fc-chimeric constructs.

#### X. TNF receptor-IgG Fc fusion protein

The nucleotide and amino acid sequences of the 75 kDa human TNF receptor are set forth herein as SEQ ID NO:31 and SEQ ID NO:32, respectively (Figure 82A and 82B, respectively). The amino acid sequences of the light and heavy variable regions of chimeric anti-HER2 are set forth as SEQ ID NO:35 and SEQ ID NO:36, respectively (Figure 83A and

83B, respectively). The amino acid sequences of the heavy and light variable regions of chimeric anti-RSV are set forth as SEQ ID NO:38 and SEQ ID NO:37, respectively (Figure 84A and 84B, respectively). The amino acid sequences of the non-human variable regions of anti-TNF are set forth herein as SEQ ID NO:41 and SEQ ID NO:42, respectively (Figure 85A and 85B, respectively). The nucleotide and amino acid sequence of the Fc portion of human IgG is set forth as SEQ ID NO:49 and SEQ ID NO:50 (Figure 86A and 86B, respectively).

A remodeled chimeric ENBREL™ may be administered to a patient selected from the group consisting of a patient having rheumatoid arthritis and a patient having polyarticular-course juvenile arthritis. A remodeled chimeric ENBREL™ may also be administered to an arthritis patient to reduce signs, symptoms, or structural damage in the patient. Preferably, the patient is a human patient.

A remodeled Synagis™ antibody may be administered to a patient to immunize the patient against infection by respiratory syncytial virus (RSV). A remodeled Synagis™ antibody may also be administered to a patient to prevent or reduce the severity of a lower respiratory tract disease caused by RSV. Preferably, the patient is a human patient.

#### Y. MAb anti-glycoprotein IIb/IIIa

The amino acid sequences of a murine anti-glycoprotein IIb/IIIa antibody variable regions are set forth in SEQ ID NO:52 (murine mature variable light chain, Figure 87) and SEQ ID NO: 54 (murine mature variable heavy chain, Figure 88). These murine sequences can be combined with human IgG amino acid sequences SEQ ID NO:51 (human mature variable light chain, Figure 89), SEQ ID NO: 53 (human mature variable heavy chain, Figure 90), SEQ ID NO: 55 (human light chain, Figure 91) and SEQ ID NO: 56 (human heavy chain, Figure 92) according to the procedures found in U.S. Patent No. 5,777,085 to create a chimeric humanized murine anti-glycoprotein IIb/IIIa antibody. Other anti-glycoprotein IIb/IIIa humanized antibodies are found in U.S. Patent No. 5,877,006. A cell line expressing the anti-glycoprotein IIb/IIIa MAb 7E3 can be commercially obtained from the ATCC (Manassas, VA) as accession no. HB-8832.

#### Indications for selected antibodies

A remodeled Reopro™ may be administered to a patient selected from the group consisting of a patient undergoing percutaneous coronary intervention and a patient having unstable angina, wherein the patient is scheduled for percutaneous coronary intervention within 24 hours. A remodeled Reopro™ may also be administered to a patient undergoing percutaneous coronary intervention to reduce or prevent a cardiac ischemic complication in the patient. Preferably, the patient is a human patient.

A remodeled Herceptin™ may be administered to a patient having metastatic breast cancer that overexpresses the HER2 protein. Preferably, the patient is a human patient.

A remodeled Remicade™ antibody may be administered to a patient selected from the group consisting of a patient having rheumatoid arthritis, a patient having Crohn's disease, and a patient having fistulizing Crohn's disease. A remodeled Remicade™ antibody may also be administered to a rheumatoid arthritis patient to reduce signs and symptoms of rheumatoid arthritis in the patient. A remodeled Remicade™ antibody may also be administered to a Crohn's disease patient to reduce signs and symptoms of Crohn's disease in the patient. Preferably, the patient is a human patient.

#### Z. MAb anti-CD20

The nucleic acid and amino acid sequences of a chimeric anti-CD20 antibody are set forth in SEQ ID NO: 59 (nucleic acid sequence of murine variable region light chain, Figure 93A), SEQ ID NO:60 (amino acid sequence of murine variable region light chain, Figure 93B), SEQ ID NO:61 (nucleic acid sequence of murine variable region heavy chain, Figure 94A) and SEQ ID NO:62 (amino acid sequence of murine variable region heavy chain, Figure 94B). In order to humanize a murine antibody, the TCAE 8 (SEQ ID NO:57, Figure 95A – 95E), which contains the human IgG heavy and light constant domains, may be conveniently used. By cloning the above murine variable region encoding DNA into the TCAE 8 vector according to instructions given in U.S. Patent No. 5,736,137, a vector is created (SEQ ID NO: 58, Figure 96A – 96E) which when transformed into a mammalian cell line, expresses a chimeric anti-CD20 antibody. Other humanized anti-CD20 antibodies are found in U.S. Patent No. 6,120,767. A cell line expressing the anti-CD20 MAb C273 can be commercially obtained from the ATCC (Manassas, VA) as accession no. HB-9303.

The skilled artisan will readily appreciate that the sequences set forth herein are not exhaustive, but are rather examples of the variable regions, receptors, and other binding moieties of chimeric antibodies. Further, methods to construct chimeric or "humanized" antibodies are well known in the art, and are described in, for example, U.S. Patent No. 6,329,511 and U.S. Patent No. 6,210,671. Coupled with the present disclosure and methods well known throughout the art, the skilled artisan will recognize that the present invention is not limited to the sequences disclosed herein.

The expression of a chimeric antibody is well known in the art, and is described in detail in, for example, U.S. Patent No. 6,329,511. Expression systems can be prokaryotic, eukaryotic, and the like. Further, the expression of chimeric antibodies in insect cells using a baculovirus expression system is described in Putlitz et al. (1990, Bio/Technology 8:651-654). Additionally, methods of expressing a nucleic acid encoding a fusion or chimeric protein are well known in the art, and are described in, for example, Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York).

Determining the function and biological activity of a chimeric antibody produced according to the methods of the present invention is a similarly basic operation for one of skill in the art. Methods for determining the affinity of an antibody by competition assays are detailed in Berzofsky (J. A. Berzofsky and I. J. Berkower, 1984, in Fundamental Immunology (ed. W. E. Paul), Raven Press (New York), 595). Briefly, the affinity of the chimeric antibody is compared to that of the monoclonal antibody from which it was derived using a radio-iodinated monoclonal antibody.

A remodeled anti-CD20 antibody may be administered to a patient having relapsed or refractory low grade or follicular, CD20-positive, B-cell non-Hodgkin's lymphoma. Preferably, the patient is a human patient.

## VII. Pharmaceutical Compositions

In another aspect, the invention provides a pharmaceutical composition. The pharmaceutical composition includes a pharmaceutically acceptable diluent and a covalent conjugate between a non-naturally-occurring, water-soluble polymer, therapeutic moiety or biomolecule and a glycosylated or non-glycosylated peptide. The polymer, therapeutic

moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide and the polymer, therapeutic moiety or biomolecule.

Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in  
5 *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, *Science* 249:1527-1533 (1990).

The pharmaceutical compositions may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial,  
10 intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose,  
15 sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

Commonly, the pharmaceutical compositions are administered parenterally, *e.g.*,  
20 intravenously. Thus, the invention provides compositions for parenteral administration which comprise the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, *e.g.*, water, buffered water, saline, PBS and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents,  
25 wetting agents, detergents and the like.

These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more  
30 preferably from 5 to 9 and most preferably from 7 and 8.

In some embodiments the peptides of the invention can be incorporated into liposomes formed from standard vesicle-forming lipids. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9: 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028. The targeting of liposomes using  
5 a variety of targeting agents (*e.g.*, the sialyl galactosides of the invention) is well known in the art (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044).

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or  
10 derivatized lipophilic compounds, such as lipid-derivatized peptides of the invention.

Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The carbohydrates of the invention may be attached to a lipid molecule before the liposome is formed using methods known to those  
15 of skill in the art (*e.g.*, alkylation or acylation of a hydroxyl group present on the carbohydrate with a long chain alkyl halide or with a fatty acid, respectively). Alternatively, the liposome may be fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion, which is firmly embedded and anchored in the membrane. It must also  
20 have a reactive portion, which is chemically available on the aqueous surface of the liposome. The reactive portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent or carbohydrate, which is added later. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector  
25 molecule which is in the membrane with the target agent or carbohydrate which is extended, three dimensionally, off of the vesicle surface. The dosage ranges for the administration of the peptides of the invention are those large enough to produce the desired effect in which the symptoms of the immune response show some degree of suppression. The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age,  
30 condition, sex and extent of the disease in the animal and can be determined by one of skill in



the art. The dosage can be adjusted by the individual physician in the event of any counterindications.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to conjugate, complex or adsorb the peptide. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino carboxymethylcellulose, and protamine sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the peptide into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly (lactic acid) or ethylene vinylacetate copolymers.

In order to protect peptides from binding with plasma proteins, it is preferred that the peptides be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly (methymethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such teachings are disclosed in Remington's Pharmaceutical Sciences (16th Ed., A. Oslo, ed., Mack, Easton, Pa., 1980).

The peptides of the invention are well suited for use in targetable drug delivery systems such as synthetic or natural polymers in the form of macromolecular complexes, nanocapsules, microspheres, or beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes, and resealed erythrocytes. These systems are known collectively as colloidal drug delivery systems. Typically, such colloidal particles containing the dispersed peptides are about 50 nm-2  $\mu$ m in diameter. The size of the colloidal particles allows them to be administered intravenously such as by injection, or as an aerosol. Materials used in the preparation of colloidal systems are typically sterilizable via filter sterilization, nontoxic, and biodegradable, for example albumin, ethylcellulose, casein, gelatin, lecithin, phospholipids, and soybean oil. Polymeric colloidal systems are prepared by a process similar to the coacervation of microencapsulation.

In an exemplary embodiment, the peptides are components of a liposome, used as a targeted delivery system. When phospholipids are gently dispersed in aqueous media, they

swell, hydrate, and spontaneously form multilamellar concentric bilayer vesicles with layers of aqueous media separating the lipid bilayer. Such systems are usually referred to as multilamellar liposomes or multilamellar vesicles (MLVs) and have diameters ranging from about 100 nm to about 4  $\mu$ m. When MLVs are sonicated, small unilamellar vesicles (SUVs) with diameters in the range of from about 20 to about 50 nm are formed, which contain an aqueous solution in the core of the SUV.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and are saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine.

In preparing liposomes containing the peptides of the invention, such variables as the efficiency of peptide encapsulation, lability of the peptide, homogeneity and size of the resulting population of liposomes, peptide-to-lipid ratio, permeability instability of the preparation, and pharmaceutical acceptability of the formulation should be considered. Szoka, et al, *Annual Review of Biophysics and Bioengineering*, 9: 467 (1980); Deamer, et al., in *LIPOSOMES*, Marcel Dekker, New York, 1983, 27; Hope, et al., *Chem. Phys. Lipids*, 40: 89 (1986)).

The targeted delivery system containing the peptides of the invention may be administered in a variety of ways to a host, particularly a mammalian host, such as intravenously, intramuscularly, subcutaneously, intra-peritoneally, intravascularly, topically, intracavitarily, transdermally, intranasally, and by inhalation. The concentration of the peptides will vary upon the particular application, the nature of the disease, the frequency of administration, or the like. The targeted delivery system-encapsulated peptide may be provided in a formulation comprising other compounds as appropriate and an aqueous physiologically acceptable medium, for example, saline, phosphate buffered saline, or the like.

The compounds prepared by the methods of the invention may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of

inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the compounds can be labeled with  $^{125}\text{I}$ ,  $^{14}\text{C}$ , or tritium.

## EXPERIMENTAL EXAMPLES

5 The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these Examples, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

10 The materials and methods used in the experiments presented in this Example are now described.

### A. General Procedures

#### 1. Preparation of CMP-SA-PEG

This example sets forth the preparation of CMP-SA-PEG.

**Preparation of 2-(benzyloxycarboxamido)-glycylamido-2-deoxy-D-mannopyranose.** N-benzyloxycarbonyl-glycyl-N-hydroxysuccinimide ester (3.125 g, 10.2 mmol) was added to a solution containing D-mannosamine-HCl (2 g, 9.3 mmol) and triethylamine (1.42 mL, 10.2 mmol) dissolved in MeOH (10 mL) and H<sub>2</sub>O (6 mL). The reaction was stirred at room temperature for 16 hours and concentrated using rotoevaporation. Chromatography (silica, 10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) yielded 1.71 g (50% yield) of product as a white solid:  $R_f = 0.62$  (silica; CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O, 6/4/1);  $^1\text{H}$  NMR (CD<sub>3</sub>OD, 500 MHz)  $\delta$  3.24-3.27 (m, 2H), 3.44 (t, 1H), 3.55 (t, 1H), 3.63-3.66 (m, 1H), 3.76-3.90 (m, 6H), 3.91 (s, 2H), 4.0 (dd, 2H), 4.28 (d, 1H,  $J = 4.4$ ), 4.41 (d, 1H,  $J = 3.2$ ), 5.03 (s, 1H), 5.10 (m, 3H), 7.29-7.38 (m, 10H).

**Preparation of 5-(N-benzyloxycarboxamido)glycylamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosurionate.** 2-(N-Benzyloxycarboxamido) glycylamide-2-deoxy-D-mannopyranose (1.59 g, 4.3 mmol) was dissolved in a solution of 0.1 M HEPES (12 mL, pH 7.5) and sodium pyruvate (4.73 g, 43 mmol). Neuraminic acid aldolase (540 U of enzyme in 45 mL of a 10 mM phosphate buffered solution containing 0.1 M NaCl at pH 6.9) and the reaction mixture was heated to 37°C for 24 hr. The reaction mixture was then centrifuged

and the supernatant was chromatographed (C18 silica, gradient from H<sub>2</sub>O (100%) to 30% MeOH/water). Appropriate fractions were pooled, concentrated and the residue chromatographed (silica, gradient from 10% MeOH/ CH<sub>2</sub>Cl<sub>2</sub> to CH<sub>2</sub>Cl<sub>2</sub>/MeOH/ H<sub>2</sub>O 6/4/1). Appropriate fractions were collected, concentrated and the residue resuspended in water.

- 5 After freeze-drying, the product (1.67 g, 87% yield) was obtained as a white solid:  $R_f = 0.26$  (silica, CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 6/4/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.82 (t, 1H), 2.20 (m, 1H), 3.49 (d, 1H), 3.59(dd, 1H), 3.67-3.86 (m, 2H), 3.87(s, 2H), 8.89-4.05 (m, 3H), 5.16 (s, 2H), 7.45 (m, 5H).

**Preparation of 5-glycylamido-3,5-dideoxy-D-glycero-D-galacto-2-**

- 10 **nonulopyranosuronate.** 5-(N-Benzyloxycarboxamido)glycylamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosuronate (1.66 g, 3.6 mmol) was dissolved in 20 mL of 50% water/methanol. The flask was repeatedly evacuated and placed under argon and then 10% Pd/C (0.225 g) was added. After repeated evacuation, hydrogen (about 1 atm) was then added to the flask and the reaction mixture stirred for 18 hr. The reaction mixture was
- 15 filtered through celite, concentrated by rotary evaporation and freeze-dried to yield 1.24 g (100% yield) of product as a white solid:  $R_f = 0.25$  (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.83 (t, 1H, J = 9.9), 2.23 (dd, 1H, J = 12.9, 4.69), 3.51-3.70 (m, 2H), 3.61(s, 2H), 3.75-3.84 (m, 2H), 3.95-4.06(m, 3H).

**Preparation of cytidine-5'-monophosphoryl-[5-(N-fluorenylmethoxy-**

- 20 **carboxamido)glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate].** A solution containing 5-glycylamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosuronate (0.55 g, 1.70 mmol) dissolved in 20 mL H<sub>2</sub>O was added to a solution of Tris (1.38 g, 11.4 mmol), 1 M MgCl<sub>2</sub> (1.1 mL) and BSA (55 mg). The pH of the solution was adjusted to 8.8 with 1M NaOH (2 mL) and CTP-2Na<sup>+</sup> (2.23 g, 4.2 mmol) was added.
- 25 The reaction mixture pH was controlled with a pH controller which delivered 1 M NaOH as needed to maintain pH 8.8. The fusion protein (sialyltransferase/CMP-neuraminic acid synthetase) was added to the solution and the reaction mixture was stirred at room temperature. After 2 days, an additional amount of fusion protein was added and the reaction stirred an additional 40 hours. The reaction mixture was precipitated in EtOH and the
- 30 precipitate was washed 5 times with cold EtOH to yield 2.3 grams of a white solid. About 1.0 g of the crude product was dissolved in 1,4-dioxane (4 mL), H<sub>2</sub>O (4 mL) and saturated

NaHCO<sub>3</sub> (3 mL) and a solution of FMOC-Cl (308 mg, 1.2 mmol) dissolved in 2 ml dioxane was added dropwise. After stirring for 16 hr at room temperature, the reaction mixture was concentrated to about 6 mL by rotary evaporation and purified using chromatography (C18 silica, gradient 100% H<sub>2</sub>O to 30% MeOH/ H<sub>2</sub>O). Appropriate fractions were combined and concentrated. The residue was dissolved in water and freeze-dried to yield 253 mg of a white solid:  $R_f$  = 0.50 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.64 (dt, 1H, J = 12.0, 6.0), 2.50 (dd, 1H, J = 13.2, 4.9), 3.38 (d, J = 9.67, 1H), 3.60 (dd, J=11.65, 6.64, 1H), 3.79 (d, J=4.11, 1H), 3.87 (dd, J= 12.24, 1.0, 1H), 3.97 (m, 2H), 4.07 (td, J = 10.75, 4.84, 1H), 4.17 (dd, J = 10.68, 1.0, 1 H), 4.25 (s, 2H), 4.32 (t, J =4.4, 1H), 4.37 (t, J=5.8 1H), 4.6-4.7 (m, obscured by solvent peak), 5.95 (d, J = 4, 1 H), 6.03 (d, J = 7.4, 1H), 7.43-7.53 (m, 3H), 7.74 (m, 2H), 7.94 (q, J = 7, 3H) . MS (ES); calc. for C<sub>35</sub>H<sub>42</sub>N<sub>5</sub>O<sub>18</sub>P ([M-H]<sup>-</sup>), 851.7; found 850.0.

**Preparation of cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate).** Diisopropylamine (83  $\mu$ L, 0.587  $\mu$ mol) was added to a solution of cytidine-5'-monophosphoryl-[5-(N-fluorenyl-methoxycarboxamido)glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate] (100 mg, 0.117 mmol) dissolved in water (3 mL) and methanol (1 mL). The reaction mixture was stirred 16 hr at room temperature and the reaction methanol removed from the reaction mixture by rotary evaporation. The crude reaction mixture was filtered through a C18 silica gel column using water and the effluent was collected and freeze-dried to yield (87 mg, 100%) of product as a white solid:  $R_f$  = 0.21 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.66 (td, 1H, J =5.3), 2.50 (dd, 1H, J = 13.2, 4.6), 3.43 (d, J = 9.58, 1H), 3.63 (dd, J = 11.9, 6.44, 1H), 3.88 (dd, J = 11.8, 1.0, 1H), 3.95 (td, J= 9.0, 2.3, 1H), 4.10 (t, J = 10.42, 1H), 4.12 (td, J = 10.34, 4.66, 1 H), 4.18 (d, J = 10.36, 1H), 4.24 (m, 2H), 4.31 (t, J=4.64, 1H), 4.35 (t, 1H), 6.00 (d, J = 4.37, 1 H), 6.13 (d, J = 7.71, 1H), 7.98 (d, J=7.64, 1H). MS (ES); calc. for C<sub>21</sub>H<sub>32</sub>N<sub>5</sub>O<sub>11</sub>P ([M-H]<sup>-</sup>), 629.47; found 627.9.

**Preparation of cytidine-5'-monophosphoryl-[5-(N-methoxy-polyoxyethylene-(1 kDa)-3-oxypropionamido)-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate].** Benzyltriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP, 21 mg, 48  $\mu$ mol) was added to a solution of methoxypolyoxyethylene-(1 kDa average molecular weight)-3-oxypropionic acid (48 mg, 48

5  $\mu\text{mol}$ ) dissolved in anhydrous DMF (700  $\mu\text{L}$ ) and triethylamine (13  $\mu\text{L}$ , 95  $\mu\text{mol}$ ). After 30 min, a solution containing cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate) (30 mg, 48  $\mu\text{mol}$ ), water (400  $\mu\text{L}$ ) and triethylamine (13  $\mu\text{L}$ , 95  $\mu\text{mol}$ ) was added. This solution was stirred 20 min at room temperature and then chromatographed (C18 silica, gradient of methanol/water). Appropriate fractions were collected, concentrated, the residue dissolved in water and freeze-dried to afford 40 mg (50% yield) of a white solid:  $R_f = 0.36$  (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.66 (td, 1H, J=5.3), 2.50 (dd, 1H, J=13.2, 4.6), 2.64 (t, J=5.99, 3H) 3.43 (d, J=9.58, 1H), 3.63 (m, 1H), 3.71 (s, 70H), 3.79 (m, obscured by 3.71 peak), 3.82 (t, J=6.19, 1H) 3.88 (dd, J=11.8, 1.0, 1H), 3.95 (td, J=9.0, 2.3, 1H), 3.98 (t, J=5.06, 1H), 4.12 (td, J=10.34, 4.66, 1H), 4.18 (d, J=10.36, 1H), 4.23 (d, J=4.85, 2H), 4.31 (t, J=4.64, 1H), 4.35 (t, 1H), 6.00 (d, J=4.55, 1H), 6.13 (d, J=7.56, 1H), 7.98 (d, J=7.54, 1H). MS (MALDI), observe [M-H]; 1594.5, 1638.5, 1682.4, 1726.4, 1770.3, 1814.4, 1858.2, 1881.5, 1903.5, 1947.3.

15 **Preparation of cytidine-5'-monophosphoryl-[5-(N-methoxy-polyoxyethylene-(10 kDa)-oxycarboxamido)-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate].** Cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate) (2.5 mg, 4  $\mu\text{mol}$ ) and water (180  $\mu\text{L}$ ) was added to a solution of (Methoxypolyoxyethylene-(10 kDa, average molecular weight)-oxycarbonyl-
 20 (N-oxybenzotriazole) ester (40 mg, 4  $\mu\text{mol}$ ) in anhydrous DMF (800  $\mu\text{L}$ ) containing triethylamine (1.1  $\mu\text{L}$ , 8  $\mu\text{mol}$ ) and the reaction mixture stirred for 1 hr at room temperature. The reaction mixture was then diluted with water (8 mL) and was purified by reversed phase flash chromatography (C18 silica, gradient of methanol/water). Appropriate fractions were combined, concentrated, the residue dissolved in water and freeze-dried yielding 20 mg (46%
 25 yield) of product as a white solid:  $R_f = 0.35$  (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.66 (td, 1H), 2.50 (dd, 1H), 2.64 (t, 3H) 3.55-3.7 (m, obscured by 3.71 peak), 3.71 (s, 488H), 3.72-4.0 (m, obscured by 3.71 peak), 4.23 (m, 3H), 4.31 (t, 1H), 4.35 (t, 1H), 6.00 (d, J=4.77, 1H), 6.12 (d, J=7.52, 1H), 7.98 (d, J=7.89, 1H). MS (MALDI), observe [M-CMP+Na]; 10780.

30

## 2. Preparation of CMP-SA-PEG II

This example sets forth the general procedure for making CMP-SA-PEG, and specific procedures for making CMP-SA-PEG (1 kDa) and CMP-SA-PEG (20 kDa).

**General procedures Preparing Cytidine-5'-monophosphoryl-(5-glycyamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate).** Cytidine-5'-monophosphoryl-(5-glycyamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate (870 mg, 1.02 mmol) was dissolved in 25 mL of water and 5.5 mL of 40 wt% dimethylamine solution in H<sub>2</sub>O was added. The reaction was stirred for 1 hr and the excess dimethyl amine was then removed by rotary evaporation. The aqueous solution was filtered through a C-18 silica gel column and the column was washed with water. The eluants were combined and lyophilized to afford 638 mg (93%) of a white solid.  $R_f = 0.10$  (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH; 7/2/1). <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.66 (td, 1H, J = 5.3), 2.50 (dd, 1H, J = 13.2, 4.6), 3.43 (d, J = 9.58, 1H), 3.63 (dd, J = 11.9, 6.44, 1H), 3.88 (dd, J = 11.8, 1.0, 1H), 3.95 (td, J = 9.0, 2.3, 1H), 4.10 (t, J = 10.42, 1H), 4.12 (td, J = 10.34, 4.66, 1H), 4.18 (d, J = 10.36, 1H), 4.24 (m, 2H), 4.31 (t, J = 4.64, 1H), 4.35 (t, 1H), 6.00 (d, J = 4.37, 1H), 6.13 (d, J = 7.71, 1H), 7.98 (d, J = 7.64, 1H). MS (ES); calc. for C<sub>21</sub>H<sub>32</sub>N<sub>5</sub>O<sub>11</sub>P ([M-H]<sup>-</sup>), 629.47; found 627.9.

**General procedures for Preparing CMP-SA-PEG using mPEG-(p-nitrophenol)carbonate.** Cytidine-5'-monophosphoryl-(5-glycyamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate) (175 mg, 0.259 mMol) was dissolved in a mixture of water, pH 8.5, and DMF or THF (in a ratio of 1:2). The mPEG-nitrophenol carbonate (2 to 20 kDa mPEG's) (0.519 mMole) was added in several portions over 8 hr at room temperature and the reaction mixture was stirred at room temperature for 3 days. When complete, water (40 ml) and 1.5 ml of NH<sub>4</sub>OH (29% aqueous solution) were added. The yellow reaction mixture was stirred for another 2 hr and then concentrated by rotary evaporation. The reaction mixture was then diluted with water (pH 8.5) to about 500 ml volume and was purified by reversed phase flash chromatography (Biotage 40M, C18 silica column) with a gradient of methanol/water. Appropriate fractions were combined and concentrated to afford the products as white solids.  $R_f$  (silica; 1-propanol / water / 29%NH<sub>4</sub>OH; 7 / 2 / 1); (2 kDa PEG) = 0.31; (5 kDa PEG) = 0.33; (10 kDa PEG) = 0.36; (20 kDa PEG) = 0.38 (TLC silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); MS (MALDI), observe [M-CMP+Na]; (2 kDa) = 2460; (5 kDa) = 5250; (10 kDa) = 10700; (20 kDa) = 22500.

**Preparation of Cytidine-5'-monophosphoryl-[5-(N-fluorenylmethoxycarboxamido)-glycylamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosurionate].** Sodium pyruvate (2.4 g, 218 mmol), HEPES buffer (0.25 M, pH 7.34) and 1.0 g (22 mmol) of Fmoc-glycylmannosamide were mixed in a 150 mL

5 polycarbonate bottle. A neuraminic acid aldolase solution (19 mL, ~ 600 U) was then added and the reaction mixture was incubated at 30 °C on an orbital shaker. After 23 hours, Thin layer chromatography (TLC) indicated that approximately 75% conversion to product had occurred. The CTP (1.72 g, 33 mmol) and 0.1 M of MnCl<sub>2</sub> (6 mL) were then added to the reaction mixture. The pH was adjusted to 7.5 with 1 M NaOH (5.5 mL) and a solution

10 containing CMP-neuraminic acid synthetase (*Neisseria*) was added (25 mL, 386 U). The reaction was complete after 24 hrs and the reaction mixture was chromatographed (C-18 silica, gradient from H<sub>2</sub>O (100%) to 10% MeOH/H<sub>2</sub>O). Appropriate fractions were recombined, concentrated and lyophilized to afford a white solid, R<sub>f</sub> (IPA/ H<sub>2</sub>O/NH<sub>4</sub>OH, 7/2/1) = 0.52. <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz) δ 1.64 (dt, 1H, J = 12.0, 6.0), 2.50 (dd, 1H, J = 13.2, 4.9), 3.38 (d, J = 9.67, 1H), 3.60 (dd, J=11.65, 6.64, 1H), 3.79 (d, J=4.11, 1H), 3.87 (dd, J= 12.24, 1.0, 1H), 3.97 (m, 2H), 4.07 (td, J = 10.75, 4.84, 1H), 4.17 (dd, J = 10.68, 1.0, 1 H), 4.25 (s, 2H), 4.32 (t, J = 4.4, 1H), 4.37 (t, J=5.8 1H), 4.6-4.7 (m, obscured by solvent peak), 5.95 (d, J = 4, 1 H), 6.03 (d, J = 7.4, 1H), 7.43-7.53 (m, 3H), 7.74 (m, 2H), 7.94 (q, J = 7, 3H). MS (ES); calc. for C<sub>35</sub>H<sub>42</sub>N<sub>5</sub>O<sub>18</sub>P ([M-H]<sup>-</sup>), 850.7; found 850.8.

20 **Preparation of Cytidine-5'-monophosphoryl-[5-(N-methoxypolyoxyethylene-(1 kDa)-3-oxypropionamido)-glycylamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosurionate].** Methoxypolyoxyethylene-(1 kDa average molecular weight)-3-oxypropionate-N-succinimidyl ester (52 mg, 52 μmol) dissolved in anhydrous DMF (450 μL) and triethylamine (33 μL, 238 μmol). Cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosurionate) (30 mg, 48 μmol) was added as a

25 solid. Water, pH 8 (330 μL) was added and after 30 min, an additional 28 mg of NHS-activated PEG was added. After an additional 5 min, the reaction mixture was chromatographed (C-18 silica, gradient of methanol/water), and appropriate fractions were concentrated to afford 32 mg (40% yield) of a white solid, R<sub>f</sub> = 0.31 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH

30 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz) δ 1.66 (td, 1H, J = 5.3), 2.50 (dd, 1H, J = 13.2, 4.6), 2.64 (t,



J=5.99, 3H) 3.43 (d, J = 9.58, 1H), 3.63 (m, 1H), 3.71 (s, 70H), 3.79 (m, obscured by 3.71 peak), 3.82 (t, J=6.19, 1H) 3.88 (dd, J = 11.8, 1.0, 1H), 3.95 (td, J= 9.0, 2.3, 1H), 3.98 (t, J= 5.06, 1H), 4.12 (td, J = 10.34, 4.66, 1 H), 4.18 (d, J = 10.36, 1H), 4.23 (d, J=4.85, 2H), 4.31 (t, J=4.64, 1H), 4.35 (t, 1H), 6.00 (d, J = 4.55, 1 H), 6.13 (d, J = 7.56, 1H), 7.98 (d, J=7.54, 1H). MS (MALDI), observe [(M-CMP)-H]; 1506.4, 1550.4, 1594.5, 1638.5, 1682.4, 1726.4, 1770.3, 1814.4, 1858.2.

**Preparation of Cytidine-5'-monophosphoryl-{5-[N-(2,6-dimethoxypolyoxyethylene-(20 kDa)-3oxypropionamidyl-lysylamido)-glycylamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosurionate].** The 2,6-Di-[methoxypolyoxyethylene-(20 kDa average molecular weight)-3-oxypropionamidyl]-lysylamido-N-succinimidyl ester (367 mg, 9 μmol) was dissolved in anhydrous THF (7 mL) and triethylamine (5 μL, 36 μmol). Cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosurionate) (30 mg, 48 μmol) was dissolved in 1.0 mL of water, and added to the reaction mixture. The reaction was stirred for 4 hours at room temperature and was then chromatographed (HPLC, Waters Xterra RP8, gradient from water/NH<sub>4</sub>OH, 100% to 20% methanol/water/NH<sub>4</sub>OH at 1 mL/min) to afford a white solid with a R<sub>t</sub> = 22.8 min. MS (MALDI), observe [(M-CMP)-H]; 43027.01 (40,000 – 45,500).

### 3. Preparation of UDP-Gal-PEG.

This example sets forth the general procedure for making UDP-Gal-PEG. Methoxypolyoxyethylenepropionate N-hydroxysuccinimide ester (mPEG-SPA, MW 1,000) 348 mg in THF (0.5 mL) was added to a solution of 25 mg of galactosamine-1-phosphate in 1 ml of water, followed by the addition of 67 μL triethylamine. The resulting mixture was stirred at room temperature for 17 hr. Concentration at reduce pressures provided a crude reaction mixture which was purified by chromatography (C-18 silica, using a step gradient of 10%, 20%, 30%, 40% aqueous MeOH) to afford 90 mg (74%) of product after the appropriate fractions were combined and concentrated to dryness. R<sub>f</sub> = 0.5 (silica, Propanol/H<sub>2</sub>O/NH<sub>4</sub>OH 30/20/2); MS(MALDI), observed 1356, 1400, 1444, 1488, 1532, 1576, 1620.

**[ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-2-deoxy-2-(methoxypolyoxyethylene-propionoylamido-1 kDa)- $\alpha$ -D-galactosamine.** The 2-deoxy-2-(methoxypolyoxyethylenepropionoylamido-1 kDa)- $\alpha$ -1-monophosphate-D-galactosamine (58 mg) was dissolved in 6 mL of DMF and 1.2 mL of pyridine. UMP-morpholidate (60 mg) was then  
 5 added and the resulting mixture was stirred at 70°C for 48 hr. After concentration, the residue was chromatographed (C18-silica, using a step gradient of 10%, 20%, 30%, 40%, 50%, 80% MeOH) to yield 50 mg of product after concentration of the appropriate fractions.  $R_f$  = 0.54 (silica, propanol/H<sub>2</sub>O/NH<sub>4</sub>OH 30/20/2). MS(MALDI); Observed 1485, 1529, 1618, 1706.

10 **[ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-deoxy-6-(methoxypolyoxyethylene-amino-2 kDa)- $\alpha$ -D-galactose.** [ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-carboxaldehyde- $\alpha$ -D-galactose (10 mg) was dissolved in 2 mL of 25 mM sodium phosphate buffer (pH 6.0) and treated with methoxypolyethyleneglycol amine (MW 2,000, 70 mg) and then 25  $\mu$ L of 1M NaBH<sub>3</sub>CN solution at 0°C. The resulting mixture was frozen at -20°C for three days. The reaction  
 15 mixture was chromatographed (HPLC, Water Xterra P8) using 0.015 M NH<sub>4</sub>OH as mobile phase A and MeOH as mobile phase B as eluent at the speed of 1.0 mL/min. The product was collected, and concentrated to yield a solid;  $R_t$  = 9.4 minutes.  $R_f$  = 0.27 (silica, EtOH/H<sub>2</sub>O 7/3).

**[ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-amino-6-deoxy- $\alpha$ -D-galactose.** Ammonium acetate 15 mg was added to a solution of [ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-carboxaldehyde- $\alpha$ -D-galactopyranoside (10 mg) in sodium phosphate buffer (pH 6.0). A solution of (25  $\mu$ L) 1M NaBH<sub>3</sub>CN was then added and the mixture was stirred for 24 hr. The solution was concentrated and the residue was chromatographed (sephadex G<sub>10</sub>) to afford 10 mg of a white solid,  $R_f$  = 0.62 (silica, EtOH/0.1 M NH<sub>4</sub>Ac).

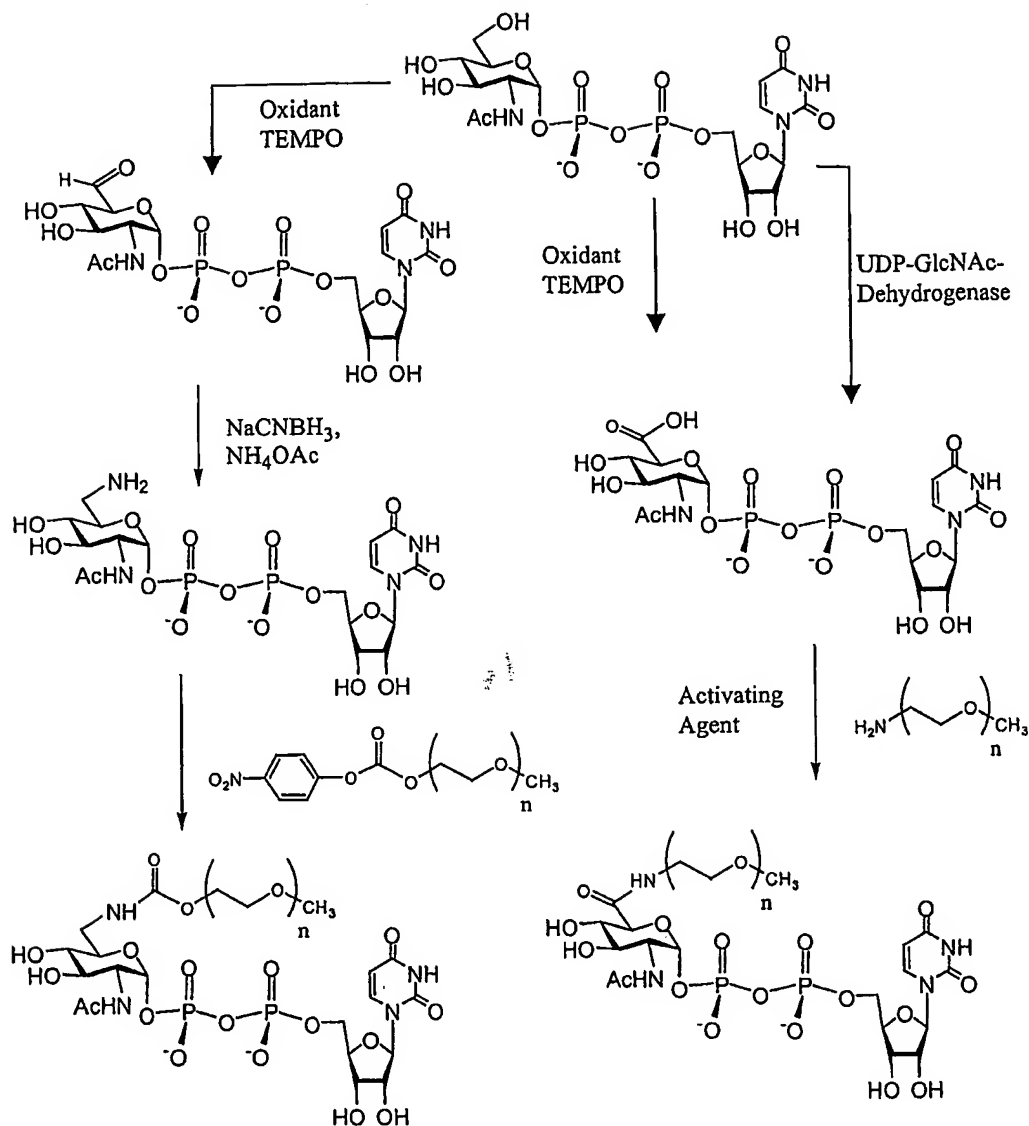
25 **[ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-deoxy-6-(methoxypolyoxyethylenepropionoylamido, ~2 kDa)- $\alpha$ -D-galactopyranoside.** [ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-amino-6-deoxy- $\alpha$ -D-galactopyranoside (5 mg) was dissolved in 1 mL of H<sub>2</sub>O. Then methoxypolyethyleneglycolpropionoyl-NHS ester (MW ~2,000, 66 mg) was added, followed by 4.6  $\mu$ L triethylamine. The resulting mixture was stirred at room temperature overnight,  
 30 and then purified on HPLC (C-8 silica) to afford the product,  $R_t$  = 9.0 min.

[ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-deoxy-6-(methoxypolyoxyethylenecarboxamido, ~2 kDa)- $\alpha$ -D-galactopyranoside. [ $\alpha$ -1-(Uridine-5'-diphosphoryl)]-6-amino-6-deoxy- $\alpha$ -D-galactopyranoside (10 mg) was mixed with methoxypolyethyleneglycolcarboxy-HOBT (MW 2000, 67 mg) in 1 mL of H<sub>2</sub>O, followed by  
5 the addition of EDC(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride 6.4 mg and 4.6  $\mu$ L triethylamine. The resulting mixture was stirred at room temperature 24 hr. The mixture was chromatographed (C-8 silica) to afford the product.

#### 4. Preparation of UDP-GlcNAc-PEG

10 This example sets forth the general procedure for making UDP-GlcNAc-PEG. On the left side of scheme 17, the protected amino sugar diphospho-nucleotide is oxidized to form an aldehyde at the 6-position of the sugar. The aldehyde is converted to the corresponding primary amine by formation and reduction of the Schiff base. The resulting adduct is contacted with the p-nitrophenol carbonate of m-PEG, which reacts with the amine,  
15 binding the m-PEG to the saccharide nucleus via an amide bond. On the right side of scheme 17 at the top, the protected amino sugar diphospho-nucleotide is treated with a chemical oxidant to form a carboxyl group at the 6-carbon of the sugar nucleus. The carboxyl group is activated and reacted with m-PEG amine, binding the m-PEG to the saccharide nucleus via an amide bond. On the right side of scheme 17 at the bottom the reactions are substantially  
20 similar to that on the top right, with the exception that the starting sugar nucleotide is contacted with an oxidizing enzyme, such as a dehydrogenase, rather than a chemical oxidant.

Scheme 17.

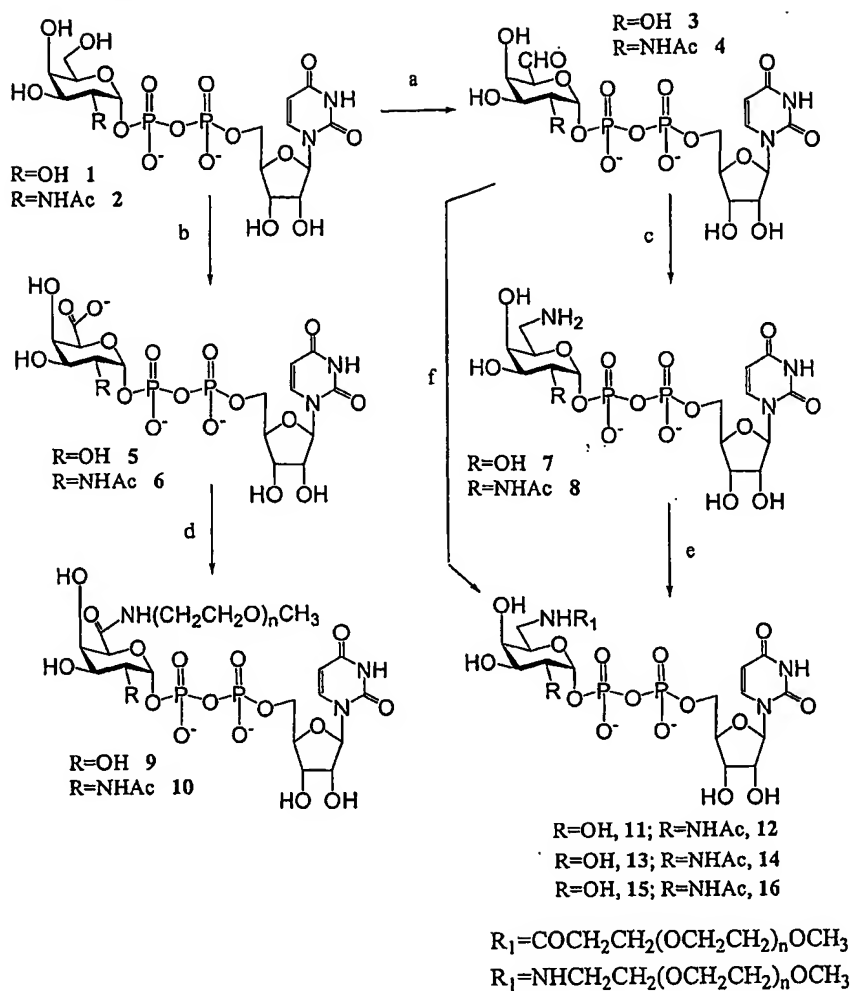


### 5. Preparation of UDP-GalNAc-PEG

- 5 This example (scheme 18) sets forth the general procedure for making UDP-GalNAc-PEG. The reaction set forth above originates with a sugar diphospho-nucleotide, in which R is either a hydroxyl 1 or a protected amine 2. In step a, the starting sugar is treated with a mixture of an oxidase and a catalase, converting the 6-position of the sugar into an aldehyde moiety (3 and 4). In step c, the aldehyde is converted to the corresponding amine (7 and 8)

by formation and reduction of a Schiff base. In step e, the amine is optionally treated with an activated m-PEG derivative, thereby acylating the amine to produce the corresponding m-PEG amide (11 and 13). Alternatively, in step f, the amine is contacted with an activated m-PEG species, such as a m-PEG active ester, thereby forming the corresponding m-PEG amide (12 and 14). In step b, the starting material is also treated with a catalase and oxidase, completely oxidizing the hydroxymethyl moiety, forming a carboxyl group at the 6-position. In step d, the carboxyl moiety is activated and subsequently converted to a m-PEG adduct (9 and 10) by reaction with a m-PEG amine intermediate. This is shown in scheme 18.

Scheme 18.

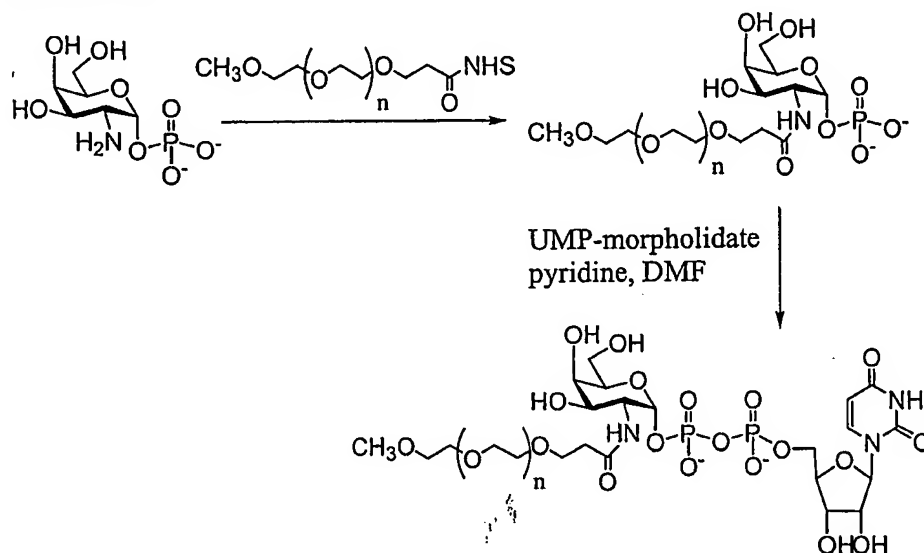


a and b): Galactose oxidase and catalase in 25 mM sodium phosphate buffer (pH 6.0); c):  $NH_4Ac$ ,  $NaBH_3CN$  in 25 mM sodium phosphate buffer (pH 6.0); d)  $CH_3(OCH_2CH_2)_nNH_2$ , EDC,  $H_2O$ ; e).  $CH_3(OCH_2CH_2)_nNH_2$ ,  $NaBH_3CN$ ,  $H_2O$  for 15 and 16; f)  $CH_3O(CH_2CH_2O)_nCH_2CH_2CONHS$ ,  $H_2O$ ,  $Et_3N$

The amino-sugar phosphate is contacted with a m-PEG N-hydroxy succinimide active ester, thereby forming the corresponding sugar-PEG-amide. The amide is contacted with UMP-morpholidate to form the corresponding active sugar diphospho-nucleotide.

5

Scheme 19.



#### 6. Synthesis of CMP-SA-Levulinate

This example sets forth the procedure for the synthesis of CMP-SA-levulinate.

10

**Preparation of 2-levulinamido-2-deoxy-D-mannopyranose.** Isobutylchloroformate (100  $\mu$ L, 0.77 mmol) was added dropwise to a solution of levulinic acid (86  $\mu$ L, 0.84 mmol), anhydrous THF (3 mL) and triethylamine (127  $\mu$ L, 0.91 mmol). This solution was stirred for 3 hours at room temperature and was then added dropwise to a solution containing D-mannosamine hydrochloride (151 mg, 0.7 mmol), triethylamine (127  $\mu$ L, 0.91 mmol), THF (2 mL) and water (2 mL). The reaction mixture was stirred 15 hours and then concentrated to dryness by rotary evaporation. Chromatography (silica, step gradient of 5-15% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) was used to isolate the product yielding 0.156 g (73% yield) of a white solid:  $R_f$  = 0.41 (silica, CHCl<sub>3</sub>/MeOH/water 6/4/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  2.23 (s, 3H), 2.24 (s, 3H), 2.57(td, J = 6.54, 3.68, 2H) 2.63 (t, J=6.71, 2H), 2.86-2.90 (m, 4H), 3.42 (m, 1H), 3.53 (t, J=9.76, 1H), 3.64 (t, J=9.43, 1H), 3.80-3.91 (m, 4H), 4.04 (dd, J = 9.79, 4.71, 1 H),

20

4.31 (dd,  $J = 4.63, 1.14$ , 1H), 4.45 (dd,  $J = 4.16, 1.13$ , 1H), 5.02 (d,  $J = 1.29$ , 1H), 5.11 (s,  $J = 1.30$ , 1H), MS (ES); calculated for  $C_{11}H_{19}NO_7$ , 277.27; found  $[M+1]$  277.9.

**Preparation of 5-levulinamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosurionate.** Sodium pyruvate (0.616 g, 5.6 mmol) and N-acetylneuraminic acid aldolase (50 U) was added to a solution of 2-levulinamido-2-deoxy-D-mannopyranose (0.156 g, 0.56 mmol) in 0.1 M HEPES (pH 7.5). The reaction mixture was heated to 37 °C for 20 hours and after freezing. The reaction mixture was then filtered through C18 silica, frozen and freeze-dried. The crude solid was purified using flash chromatography (silica, first using 10-40% MeOH/ $CH_2Cl_2$  and then  $CH_2Cl_2$ /MeOH/ $H_2O$  6/4/0.5). Appropriate fractions were combined and concentrated yielding 45 mg (80% yield) of a white solid:  $R_f = 0.15$  (silica,  $CHCl_3$ /MeOH/water 6/4/1);  $^1H$  NMR ( $D_2O$ , 500 MHz)  $\delta$  1.82 (t,  $J = 11.9$ , 1H), 2.21 (dd,  $J = 13.76, 4.84$ , 1H), 2.23 (s, 3H), 2.57 (app q,  $J = 6.6$ , 2H), 2.86-2.95 (m, 2H), 3.15-3.18 (m, 1H), 3.28-3.61 (complex, 1H), 3.60 (dd,  $J = 11.91, 6.66$ , 1H), 3.75 (td,  $J = 6.65, 2.62$ , 1H), 3.84 (dd,  $J = 11.89, 2.65$ , 1H), 3.88-4.01 (complex, 2H), 4.04 (td,  $J = 11.18, 4.67$ , 1H), MS (ES); calculated for  $C_{14}H_{23}NO_{10}$ , 365.33; found  $[M-1]$ , 363.97.

**Preparation of cytidine-5'-monophosphoryl-(5-levulinamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosurionate).** 5-Levulinamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosurionate (50 mg, 137  $\mu$ mol) was dissolved in 2 mL of 100 mM HEPES pH 7.5 buffer and 1 M  $MnCl_2$  (300  $\mu$ L; 300  $\mu$ mol) was added. CTP- $2Na^+$  (79 mg, 1.5  $\mu$ mol) was dissolved in 5 mL HEPES buffer and was added to the sugar. The sialyltransferase/CMP-neuraminic acid synthetase fusion enzyme (11 U) was added and the reaction mixture stirred at room temperature for 45 hours. The reaction mixture was filtered through a 10,000 MWCO filter and the filtrate, which contained the product of the reaction, was used directly without further purification:  $R_f = 0.35$  (silica, IPA/water/ $NH_4OH$  7/2/1).

25

## B. Glycoconjugation and GlycoPEGylation of Peptides

### $\alpha$ -Protease Inhibitor ( $\alpha$ -Antitrypsin)

#### 7. Sialylation of Recombinant Glycoproteins Antithrombin III, Fetuin and $\alpha$ 1-Antitrypsin

This example sets forth the preparation of sialylated forms of several recombinant peptides.

30



**Sialylation of Recombinant Glycoproteins Using ST3Gal III.** Several glycoproteins were examined for their ability to be sialylated by recombinant rat ST3Gal III. For each of these glycoproteins, sialylation will be a valuable process step in the development of the respective glycoproteins as commercial products.

**Reaction Conditions.** Reaction conditions were as summarized in Table 11. The sialyltransferase reactions were carried out for 24 hour at a temperature between room temperature and 37°. The extent of sialylation was established by determining the amount of <sup>14</sup>C-NeuAc incorporated into glycoprotein-linked oligosaccharides. See Table 11 for the reaction conditions for each protein.

**Table 11. Reaction conditions.**

Protein	Source	Protein Total (mg)	Protein Conc. (mg/ml)	ST (mU/mL)	ST/Protein (mU/mg)	CMP-NeuAc of "cycle" <sup>1</sup>
ATIII	Genzyme Transgenics	8.6	4.3	210	48	cycle
ATIII	Genzyme Transgenics	860	403	53	12	cycle
Asialo-fetuin	Sigma	0.4	105	20	13	10 mM
asialo-AAAT	PPL	0.4	0.5	20	20	20 mM

<sup>1</sup> "Cycle" refers to generation of CMP-NeuAc "in situ" enzymatically using standard conditions as described in specification (20 mM NeuAc and 2 mM CMP). The buffer was 0.1 M HEPES, pH 7.5.

The results presented in Table 12 demonstrate that a remarkable extent of sialylation was achieved in every case, despite low levels of enzyme used. Essentially, complete sialylation was obtained, based on the estimate of available terminal galactose. Table 12 shows the results of the sialylation reactions. The amount of enzyme used per mg of protein (mU/mg) as a basis of comparison for the various studies. In several of the examples shown, only 7-13 mU ST3Gal III per mg of protein was required to give essentially complete sialylation after 24 hours.

**Table 12. Analytical results**

Protein	Source	Terminal Gal <sup>1</sup> mol/mol	NeuAc Incorp. <sup>2</sup> mol/mol	% Rxn <sup>3</sup>	Other characterization
---------	--------	-----------------------------------	------------------------------------	--------------------	------------------------

ATIII <sup>4</sup>	Genzyme	102	104	117	None
	Transgenics				
ATIII <sup>4</sup>	Genzyme	102	1.3	108	SDS-gels: protein purity
	Transgenics				FACs: carbohydrate glycoforms
Asialo-fetuin	Sigma	802	905	116	None
asialo-AAAT <sup>5</sup>	PPL	7	7.0	100	SDS-gels: protein purity

<sup>1</sup> Terminal (exposed) Gal content on N-linked oligosaccharides determined by supplier, or from literatures values (fetuin, asialo-AAAT).

<sup>2</sup> NeuAc incorporated determined by incorporation of <sup>14</sup>C-NeuAc after separation from free radiolabeled precursors by gel filtration.

5 <sup>3</sup> The % Rxn refers to % completion of the reaction based on the terminal Gal content as a theoretical maximum.

<sup>4</sup> Antithrombin III.

<sup>5</sup>  $\alpha$ 1 Antitrypsin.

10 These results are in marked contrast to those reported in detailed studies with bovine ST6Gal I where less than 50 mU/mg protein gave less than 50% sialylation, and 1070 mU/mg protein gave approximately 85-90% sialylation in 24 hours. Paulson et al. (1977) J. Biol. Chem. 252: 2363-2371; Paulson et al. (1978) J. Biol. Chem. 253: 5617-5624. A study of rat  $\alpha$ 2,3 and  $\alpha$ 2,6 sialyltransferases by another group revealed that complete sialylation of asialo-  
15 AGP required enzyme concentrations of 150-250 mU/mg protein (Weinstein et al. (1982) J. Biol. Chem. 257: 13845-13853). These earlier studies taken together suggested that the ST6Gal I sialyltransferase requires greater than 50 mU/mg and up to 150 mU/mg to achieve complete sialylation.

This Example demonstrates that sialylation of recombinant glycoproteins using the  
20 ST3 Gal III sialyltransferase required much less enzyme than expected. For a one kilogram scale reaction, approximately 7,000 units of the ST3Gal III sialyltransferase would be needed, instead of 100,000-150,000 units that earlier studies indicated. Purification of these enzymes from natural sources is prohibitive, with yields of only 1-10 units for a large scale preparation after 1-2 months work. Assuming that both the ST6Gal I and ST3Gal III  
25 sialyltransferases are produced as recombinant sialyltransferases, with equal levels of expression of the two enzymes being achieved, a fermentation scale 14-21 times greater (or more) would be required for the ST6Gal I sialyltransferase relative to the ST3Gal III

sialyltransferase. For the ST6Gal I sialyltransferase, expression levels of 0.3 U/l in yeast has been reported (Borsig et al. (1995) Biochem. Biophys. Res. Commun. 210: 14-20).

Expression levels of 1000 U/liter of the ST3 Gal III sialyltransferase have been achieved in *Aspergillus niger*. At current levels of expression 300-450,000 liters of yeast fermentation would be required to produce sufficient enzyme for sialylation of 1 kg of glycoprotein using the ST6Gal I sialyltransferase. In contrast, less than 10 liter fermentation of *Aspergillus niger* would be required for sialylation of 1 kg of glycoprotein using the ST3Gal III sialyltransferase. Thus, the fermentation capacity required to produce the ST3Gal III sialyltransferase for a large scale sialylation reaction would be 10-100 fold less than that required for producing the ST6Gal I; the cost of producing the sialyltransferase would be reduced proportionately.

### Cri-IgG Antibody

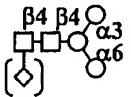
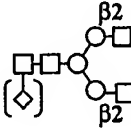
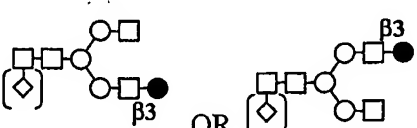
#### 8. Glyco-Remodeling of Cri-IgG1 Antibodies

This example sets forth the procedures for *in vitro* remodeling of Cri-IgG1 antibodies.

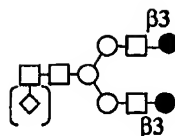
N-glycosylation at one conserved site at Asn 297 in the Fc domain of a monoclonal antibody can modulate its pharmacokinetic behavior and effector functions (Dwek et al., 1995, J. Anat. 187:279-292; Boyd et al., 1995, Mol. Immunol. 32:1311-1318; Lund et al., 1995, FASEB J. 1995, 9:115-119; Lund et al., 1996, J. Immunol. 157:4963-4969; Wright & Morrison, 1998, J. Immunol. 160:3393-3402; Flynn & Byrd, 2000, Curr. Opin. Oncol. 12:574-581). During cell culture fermentation or in certain pathological conditions, significant heterogeneity arises in the glycosylation pattern at this site. The resulting different patterns of glycosylation on the Fc domain are characterized by complex biantennary structures with zero, one, and two terminal galactose residues (G0, G1, and G2, respectively, see Table 13). The observed glycoform variations, such as the variation in terminal galactosylation, truncated N-glycoforms and bisecting modification, have been shown to influence the antibody's therapeutic properties, especially its ability to mediate targeted cell killing through complement binding and activation (Boyd et al., 1995, *supra*; Wright & Morrison, 1998, *supra*, Mimura et al., 2000, Molec. Immunol. 37:697-706; Davies et al., 2001, Biotechnol. Bioeng. 74:288-294).

In order to obtain different glycoforms of Cri-IgG1 antibodies and test their Fc effector functions, Cri-IgG1 antibodies were trimmed back stepwise using exoglycosidases to generate glycoforms lacking sialic acid (G2, G1), glycoforms lacking sialic acid and galactose (G0), and glycoforms lacking sialic acid, galactose and N-acetyl glucosamine (M3N2F), as illustrated in Table 13. These molecules were subsequently modified using different glycosyltransferases and appropriate sugars. Modification conditions were developed that resulted in the conversion of the original antibody glycan structures into different glycoforms: M3N2, GnT-I-M3N2 (the M3M2 glycoform with a GlcNAc moiety added using GnT-I), G0, Bisecting-G0 (the G0 moiety with a bisecting GlcNAc added with GnT-III), galactosylated bisecting-G0 (the bisecting-G0 glycoform with terminal galactose moieties added), G2, mono-sialylated S1( $\alpha$ 2,6)-G2 (the G2 glycoform with one terminal sialic acid moiety added using  $\alpha$ 2,6-sialyltransferase), S1( $\alpha$ 2,3)-G2 (the G2 glycoform with one terminal sialic acid moiety added using  $\alpha$ 2,3-sialyltransferase) and disialylated S2( $\alpha$ 2,3)-G2 (the G2 glycoform). After every glycoremodeling step, the glycan structures were enzymatically released from the antibody protein and were analyzed by various methods, including separation by capillary electrophoresis, 2-AA HPLC profiling and MALDI-TOF mass spectrometry.

**Table 13.** Abbreviations for glycoform structures.

Abbreviation	Glycan Structure(s)
M3N2(F)	
G0	
G1	

G2




---

◇ = fucose, □ = GlcNAc, ○ = mannose, ● = galactose

The materials and methods used in these experiments are now described.

The Cri-IgG1 Monoclonal Antibody. The Cri-IgG1 antibody was obtained from R. Jefferies, MRC Center for Immune Regulation, The Medical School, University of Birmingham, UK. The antibody is a non-recombinant antibody, and is isolated from a human myeloma. The antibody was prepared using three methods. In the first method, referred to as "DEAE," the antibody was isolated under relatively mild conditions using a DEAE ion exchange column. In the second method, referred to as "SPA," the antibody was purified on a protein A column (*Staphylococcus aureus* protein A) with a low pH elution step. In the third method, referred to as "Fc," the antibody was treated with a protease so that only the Fc portion of the antibody remained and the antigen binding domains were removed. These methods for antibody purification are well known to those of skill in the art and are not repeated in detail here.

**Affinity purification of remodeled antibodies.** Antibody, modified either by exoglycosidase or glycosyltransferase, was affinity purified on a ProA-sepharose 4-fast flow column (Amersham Bioscience, Arlington Heights, IL), eluted with 0.1 M glycine-HCl buffer, pH 2.7, and immediately neutralized with 1 M Tris, pH 9.5. The eluates were buffer-exchanged using a NAP-10 column (Amersham Bioscience, Arlington Heights, IL) to an appropriate buffer for the next step of glycosylation, such as 100 mM MES, pH 6.5 or 50 mM Tris-HCl, pH 7.2. The remodeled final products were dialyzed extensively against PBS at 4°C in Tube-O-Dialyzers™ (Chemicon International, Temecula, CA) with a MWCO of 8 kDa.

*In vitro* glycosidase treatment of Cri-antibodies. Antibody was buffer-exchanged into 50 mM Na phosphate/Citrate, pH 6.0 using NAP-10 column (Amersham Bioscience, Arlington Heights, IL). *In vitro* trimming back of sugar moieties was carried out stepwise, by contacting the antibody (5 mg/mL) with 20 mU/mg protein neuraminidase at 37°C overnight

(to remove terminal sialic acid moieties), 20 mU/mg protein  $\beta$ -galactosidase at 37°C, overnight (to remove terminal galactose moieties to result in the G0 glycoform), and/or 2 U/mg  $\beta$ -N-acetylhexosaminidase (from Jack Bean, Seikagaku, Tokyo, Japan) at 37°C, overnight (to remove terminal N-acetyl glucosamine to result in the M3N2 glycoform). The samples were affinity purified as described above.

*In vitro* glycosylation of Cri-antibodies. *In vitro* GnT1 modification was performed using 1 mg/ml of the M3N2 glycoform antibody as the substrate, and 25 mU/mg of recombinant human  $\beta$ 1,2-mannosyl-UDP-N-acetylglucosaminosyltransferase in a buffer of 100 mM MES, pH 6.5, 5 mM  $MnCl_2$ , 5 mM UDP-GlcNAc, and 0.02%  $NaN_3$  at 32°C for 24 hr. An aliquot was removed for glycan analysis, and the resulting products were affinity purified as described above.

*In vitro* modification of the bisecting-glycoform was carried out using 1 mg/ml of the M3N2 glycoform antibody as the substrate and 25 mU/mg of  $\beta$ 1,2-recombinant human mannosyl-UDP-N-acetylglucosaminosyltransferase I, 25 mU/mg of  $\beta$ 1,2-recombinant human mannosyl-UDP-N-acetylglucosaminosyltransferase II and 3.5 mU/mg of  $\beta$ 1,4-recombinant mouse mannosyl-UDP-N-acetylglucosaminosyltransferase III in a buffer of 100 mM MES pH 6.5, 10 mM  $MnCl_2$ , 5 mM UDP-GlcNAc, and 0.02%  $NaN_3$  at 32°C for 24 hrs. An aliquot was removed for glycan analysis, and the remaining product was affinity purified as described above.

*In vitro* galactosylation was performed using G0 glycoform antibody or bisecting glycoform antibody by contacting the antibody with 0.6 U/mg recombinant bovine milk  $\beta$ 1,4 galactosyltransferase in a buffer of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM UDP-galactose, 5 mM  $MnCl_2$ , at 32°C for 24 hrs. An aliquot was removed for glycan analysis, and the remaining products were affinity purified as described above.

*In vitro* sialylation was carried out using the G2 glycoform antibody (1 mg/mL) by contacting it with 0.1 U/mg ST3Gal3 or 0.1 U/mg ST6Gal1, 5 mM CMP-sialic acid, at 32°C for 24 hr in a buffer of 50 mM Tris pH 7.4, 150 mM NaCl, and 3 mM CMP-SA. An aliquot was removed for glycan analysis, and the remaining products were affinity purified as described above.

#### Glycan Analysis:

**Capillary Electrophoresis with Laser Induced Fluorescence Detection.** Buffer components and nucleotide sugars were removed from an aliquot of the glycoremodeled antibody by dilution and concentration in a Microcon™ YM-30 microconcentrator (Millipore, Bedford, MA). N-linked oligosaccharides were released from the protein by contacting it with PNGase F (Prozyme, San Leandro, CA) using the methodology provided by the manufacturer. In brief, the sample was denatured in the buffer of 50 mM sodium phosphate pH 7.5, 0.1% SDS, and 50 mM β-mercaptoethanol for 10 min at 100°C. TX100 was then added to 0.75% (v/v) as well as 10U PNGaseF/200 μg protein. After 3 hours incubation at 37°C, the protein was ethanol precipitated and the supernatant was dried down. The released free oligosaccharides were then labeled with 8-aminopyrene-1,3,6-trisulfonic acid and analyzed by capillary electrophoresis with a carbohydrate labeling and analysis kit from Beckman-Coulter, Inc. (Fullerton, CA), as indicated by the manufacturer (see also, Ma and Nashabeh, 1999, Anal. Chem. 71:5185-5192).

Capillary electrophoresis (CE) was carried out in an eCAP™ N-CHO coated Capillary (50 μm I.D., length to detector 40 cm; Beckman-Coulter, Inc., Fullerton, CA), using a P/ACE™ MDQ Glycoprotein System (Beckman-Coulter, Inc. Fullerton, CA) with Laser Induced Fluorescence Detector (Beckman-Coulter, Inc. Fullerton, CA). Samples were introduced into the cartridge by 20 psi pressure for 10 sec. and separated under 25 kV with reverse polarity for 20 min. Cartridge temperature was kept at 20°C. The electropherogram was generated by laser-induced fluorescence detection at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

Carbohydrate standards (Calbiochem®, EMD Biosciences, Inc., San Diego, CA), including M3N2 (N-linked trimannosyl core without core fucose), G0 (N-linked oligosaccharide, asialo, agalacto, biantennary with core fucose), G2 (N-linked oligosaccharide, asialo, biantennary with core fucose), and G2 without fucose, S1-G2 (mono-sialylated, galactosylated biantennary oligosaccharide without core fucose) and S2-G2 (di-sialylated, galactosylated biantennary oligosaccharide without core fucose), (from Glyko, see, ProZyme, San Leandro, CA), M3N2F (N-linked trimannosyl core with core fucose) and NGA2F (N-linked oligosaccharide asialo, agalacto, biantennary with core fucose and with bisecting GlcNAc) were labeled with 1-aminopyrene-3,6,8-trisulfonate (APTS, Beckman-

Coulter, Inc. Fullerton, CA) and used to identify the distribution of glycans released from the antibody.

2-AA HPLC. PNGaseF released glycans were labeled with 2-AA (2-anthranilic acid) according to the method described by Anumula and Dhume with slight modifications (1998, Glycobiology 8:685- 694): Reductively-aminated N-glycans were analyzed using a Shodex Asahipak NH2P-50 4D amino column (4.6 mm x 150 mm) (Showa Denko K.K., Tokyo, Japan). The two solvents used for the separation are A) 2% acetic acid and 1% tetrahydrofuran in acetonitrile and B) 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran in water.

10 To separate neutral 2AA-labeled glycans, the column was eluted isocratically with 70% A for 5 minutes, followed by a linear gradient over a period of 60 minutes going from 70% to 50% B, followed by a steep gradient over a period of 5 minutes going from 50% to 5% B and a final isocratic elution with 5% B for 10 minutes. Eluted peaks were detected using fluorescence detection with an excitation at 230 nm and detection wavelength at 420  
15 nm. In this gradient condition, the G0 glycoform will elute at about 30.5 minutes, the G1 glycoform at about 34.0 minutes and the G2 glycoform at about 37.0 minutes. Under these conditions, the presence of fucose does not change the elution time.

To separate anionic 2AA-labeled glycans, the column was eluted isocratically with 70% A for 2.5 minutes, followed by a linear gradient over a period of 97.5 min going from  
20 70% to 5% A and a final isocratic elution with 5% A for 15 minutes. Eluted peaks were detected using fluorescence detection with excitation at 230 nm and detection at 420 nm. In this gradient, neutral glycans are expected to elute between 18.00 - 29.00 minutes, glycans with one charge elute between 30.00 – 40.00 minutes, glycans with two charges elute between 43.00 – 52.00 minutes, glycans with three charges elute between 54.00 – 63.00  
25 minutes, and glycans with four charges elute between 65.00 – 74.00 minutes.

MALDI analysis of reductively-aminated N-glycans. A small aliquot of the PNGase-released N-glycans that were labeled with 2-anthranilic acid (2AA) were then dialyzed for 45 minutes on a MF-Millipore membrane filter (0.025 µm pore, 47 mm dia.), which was floating on water. The dialyzed aliquot was dried in a Speedvac™ (ThermoSavant, Holbrook, NY),  
30 redissolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (10 g/L) dissolved in water/acetonitrile (50:50).



The mixture was dried onto a MALDI target and analyzed using an Applied Biosystems DE-Pro mass spectrometer (Applied Biosystems, Inc., Foster City, CA) operated in the linear/negative-ion mode. Oligosaccharide structures were assigned based on the observed mass-to-charge ratio and literature precedence. No attempt was made to fully  
 5 characterize isobaric structures.

SDS-PAGE. To determine the stability of the glycoremodeled antibody, all the samples were analyzed by SDS-PAGE. The final products of the samples were run under non-reducing conditions using 8-16% Tris-glycine gel (Invitrogen, Carlsbad, CA). Bovine serum albumin was run under reducing condition as quantitative standards. The gel was  
 10 stained with GelCode Blue Stain Reagent (Pierce Chemical Co., Rockford, IL) for visualization.

The results of the experiments are now described.

Native glycoforms of Cri expressed in human myeloma cells. Cri-IgG1 antibody  
 15 purified from the serum of a patient having multiple myeloma contains variable glycoforms. Figure 97A-97C shows the HPLC profiles of glycans enzymatically released from Cri-IgG1 antibody. Figure 98A-98C shows the MALDI profiles of glycans enzymatically released from Cri-IgG1 antibody expressed in human myeloma cells. The major forms are under-galactosylated G0, G1, while G2 and sialylated structures are relatively minor (Table 14 and  
 20 Figure 97C). To test the impact of modified glycans on the therapeutic properties of the monoclonal antibody, Cri-IgG1 antibody was modified by performing *in vitro* exoglycosidases trimming and *in vitro* glycosylation remodeling to generate different glycoforms of this antibody.

25 **Table 14.** Relative amount of different glycoforms of human myeloma cell-expressed Cri-IgG1 separated by HPLC was calculated from the areas of individual peaks.

Criantibodies	S1G2	G2	G1	G0
DEAE			45.04	54.96
SPA	6	3.17	48.25	51.75
Fc			51.41	38.83

Initially, optimization of each step in exoglycosidases trimming and glycosylation was performed at small scale (100 µg of each).

Trimannosyl core glycoform of Cri-IgG1 Antibody (M3N2). M3N2 was created by stepwise treatment of glycosidases, including neuraminidase,  $\beta$ 1,4-galactosidase and  $\beta$ 1-2, 3, 4, 6 N-acetylhexosaminidase. To assess the removal of terminal galactose and GlcNAc on the glycoremodeled Cri-IgG1 antibody samples, a quantitative capillary electrophoresis (CE) method was used. The glycans were enzymatically released from the glycoremodeled antibody with PNGase F and were derivatized with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) at the reducing terminus. The resulting products were analyzed by CE with on-column laser-induced fluorescence detection (LIF) (Ma & Nashabeh, 1999, *supra*). Since the separation of the glycans is based on the differences in hydrodynamic size, the APTS labeled glycans migrate in order of increasing size (M3N2 < M3N2F < G0 < G1 < G2).

Figures 99A-99D show the electropherograms indicating the glycans released from glycoremodeled Cri-IgG1 antibody as well as glycan standards derivatized with APTS (Figure 99A). The glycoforms were identified by comparing their electrophoretic mobilities to the standards. The relative amount of each glycan species was calculated from the relative area percentage of each indicated peak, and the results are presented in Table 15. The M3N2F glycoform represents 91% of the glycans of DEAE-Cri, 80% of the glycans of SPA-Cri, and 100% of the glycans of Fc-Cri. Incomplete removal of GlcNAc moiety resulting in the GnT-I-M3N2F glycoform (see, Table 15) was observed in the glycan structures from DEAE-Cri (8.6%) and SPA-Cri (~20%). Glycoform GnT-I-M3N2F is the M3N2F glycoform with one additional GlcNAc, such as would be added by GnT-I.

**Table 15.** The areas of individual peaks from CE profile in Fig. 99 were calculated, and relative amounts of the M3N2F and GnT-I-M3N2F glycoforms were determined.

	M3N2F		GnT-I-M3N2F	
	RT (min.)	%	RT (min.)	%
DEAE	10.133	91.4	10.842	8.6
SPA	10.133	80.01	10.842	19.99
Fc	10.133	100	10.842	0

Degalactosylated glycoform (G0). Cri-IgG1 antibody with G0 glycoforms was obtained by stepwise treatment the native Cri-IgG1 antibody with neuraminidase and  $\beta$ 1,4-galactosidase in for 24 hours for each reaction. The glycans released from the glycoremodeled antibody were analyzed by CE, HPLC and MALDI. Figure 100A shows the CE profile of the released glycans. In all three samples, only one peak was observed which was designated as the G0 glycoform based on comparison with the standards (Fig. 100A and Table 16).

**Table 16.** The relative amount of the G0 glycoform of Cri-IgG1 determined by CE and HPLC.

	CE		HPLC	
	RT (min.)	%	RT (min.)	%
DEAE	11.408	100.0	31.194	100.0
SPA	11.408	100.0	31.194	100.0
Fc	11.408	100.0	31.194	100.0

In addition to the glycan analysis provided by CE, a quantitative HPLC method was also used to determine the percent of the G0 glycoform represented by remodeled glycans of the Cri-IgG1 antibody. The glycan distribution on the glycoremodeled antibody was monitored by enzymatically releasing the glycans with PNGase F and derivatizing the released products with 2-anthranilic acid (2-AA) at the reducing terminus. The derivatized mixture was separated by HPLC on a Shodex Asahipak NH2P-50 4D column with fluorescence detection. Figures 101A-101C show the chromatograms obtained from the released glycans. HPLC results confirmed CE analysis, as only one major peak was found in all three samples. In agreement with CE and HPLC data, MALDI analysis also showed almost complete glycoremodeling to the G0 glycoform (Fig. 102A-102C).

Fully galactosylated G2 glycoform (G2). Cri-IgG antibodies were treated with neuraminidase to yield asialo-glycoforms which were also under galactosylated. These asialoglycoforms were then treated with 0.6 U/ml of bovine  $\beta$ 1,4 galactosyltransferase and a galactose donor molecule to glycoremodel the antibody to have the G2 glycoform.

The extent of terminal galactosylation was determined by glycan analysis. Only one major peak was observed in both CE and HPLC profiles (Figure 103A-103C and Fig. 104A-104C). This peak corresponds to the G2 glycoform in each case. Calculation of the percent total peak area showed almost complete (~90%) conversion to the G2 from the under galactosylated glycoforms of the original samples (see, Table 14). These results are summarized in Table 17. MALDI analysis of the glycans further supported the almost to complete glycoremodeling to the G2 glycoform in all of the samples (Fig. 105A-105C).

**Table 17.** Relative amount of G2 glycoform of remodeled Cri-IgII antibody determined by percent total peak area in CE and HPLC analysis.

	CE		HPLC	
	RT (min.)	%	RT (min.)	%
DEAE	12.94	90	31.194	100
SPA	12.94	92	31.194	90
Fc	12.94	84	31.194	89

GnT-I-glycoform (GnT-I-M3N2). The M3N2 glycoform Cri-IgG antibody was glycoremodeled to the GnT-I-M3N2 glycoform by adding one GlcNAc moiety to the molecule. The molecule was contacted with 25 mU GnT-I/mg antibody and an appropriate GlcNAc donor molecule. CE, HPLC and MALDI analysis of released glycans (Figures 106A-106D, Figures 107A-107C and Fig. 108A-108C, respectively) indicated that the original M3N2F glycoform was completely remodeled. However, only 40-60% of the modified structures were the GnT-I-M3N2 glycoform, and about 30% were the G0 glycoform. The presence of the G0 glycoform may be the result of incomplete GlcNAc trimming when making the original M3N2 form.

Bisecting glycoform (NGA2F). The M3N2 glycoform Cri-IgG antibody was glycoremodeled to the NGA2F glycoform by contacting it with a combination the three transferases, GnT-I, GnT-II and GnT-III, and an appropriate N-acetylglucosamine donor molecule. The reaction was completed in 24 hours. To determine the extent to which the bisecting-GlcNAc moiety was added to the glycans, CE analysis was used to determine the glycoforms present on the glycoremodeled antibody.

Figure 109A-109D shows the electropherograms obtained from CE analysis of the glycans released from glycoremodeled Cri-IgG1 antibody. Four peaks appeared after remodeling. A major peak migrated at the same retention time as the NGA2F standard glycoform. The three other minor peaks are likely to be the incompletely remodeled glycans.

5 For comparison, a quantitative HPLC method was also used, where the 2-AA labeled glycans eluted in order of increasing size (Gn1 < G0 < NGA2F). As shown in Figure 110A-110C, similar results were obtained from the CE analysis of the glycans. No M3N2F was found using either the CE or HPLC analysis. NGA2F glycans were the major peaks in both CE and HPLC analysis. The Gn1 and G0 glycans still remaining in the sample likely are the result of  
10 incomplete modification. Most of the original M3N2F glycoforms were remodeled by three GlcNAc moieties to the NGA2F glycoform (60~70%), about 15~18% were remodeled by the addition of two GlcNAc moieties to the G0 glycoform, and only small amount (~ 7%) were remodeled by the addition of only one GlcNAc moiety. MALDI-MS analysis of the released glycans (Figure 111A-111C) shows peaks of glycoforms with one, two or three terminal  
15 GlcNAc moieties, in agreement with CE and HPLC analysis (Figures 109 and 110). The relative amount of each glycan species was calculated from the relative area percentage of each indicated peak, and is summarized in Table 18.

**Table 18.** Relative amounts of different glycoforms from GnT-I, II, and III remodeled Cri-IgG1, as determined by CE and HPLC.

		% Peak Area			
		Retention (min.)	DEAE	SPA	Fc
CE	Peak 1	10.238	6.39	6.89	7.98
	Peak 2	10.775	15.82	14.29	17.9
	Peak 3	11.325	14.14	8.87	15.69
	Bisec.	11.625	63.65	70.04	58.43
HPLC	Peak 1	21.117	37.4	15.02	14
	Peak 2	26.817	12.9	14.24	10.15
	Peak 3	31.224	14.78	2.11	30.2

Bisec.	32.078	34.93	68.63	45.64
--------	--------	-------	-------	-------

Galactosylated Bisecting (Gal-NGA2F) glycoforms. NGA2F glycoforms of Cri-IgG1 antibodies were glycoremodeled with bovine  $\beta$ 1,4-galactosyltransferase and an appropriate galactose donor. The terminal galactose moieties were added using 0.6 U/ml of  $\beta$ 1,4 galactosyltransferase. Figure 112A-112D shows the electropherograms obtained using the 2-AA HPLC method. In brief, the glycoforms terminating in GalNAc were almost 100% galactosylated. Comparing Figure 112A to Figure 112B for DEAE Cri-IgG1, and Figure 112C to Figure 112D for Fc Cri-IgG1, the 2-AA HPLC profile of GnT-I, II and III modified glycans (Figures 112A and 112C) is modified by GalT1 so that all of the glycan peaks were shifted to elute later due to the size increase from added galactose moieties (Fig. 112B and 112D). These results were further confirmed by MALDI-MS analysis.

Sialylated (S2G2) glycoforms of Cri-IgG1. The glycoremodeled G2 glycoforms of Cri-IgG1 antibody were further remodeled using both ST3Gal3 and ST6Gal1. Figure 113A-113C shows the HPLC profile of the G2 glycoforms remodeled with ST3Gal3. Most of the G2 glycoforms were converted into S2G2 glycoforms (the G2 glycoform with 2 additional terminal sialic acid moieties; ~70%, see, Table 19), and only small amounts were the S1G2 glycoform (the G2 glycoform with 1 additional terminal sialic acid moiety; <25%, see Table 19). These results were further confirmed in the MALDI analysis shown in Figures 114A-114C. MALDI data also shows that all the G2 glycoforms were sialylated to either S2G2 or S1G2 glycoforms.

**Table 19.** Relative amounts of different glycoforms from ST3Gal3 remodeled Cri-IgG1 as determined by HPLC.

	RT (min.)	DEAE	SPA	Fc
S1G2	36.7	25.6	24.83	23.39
	46.9	4.12	6.83	
S2G2	49.4	58.93	50.68	61.88
	52.19	9.1	7.56	6.07

By comparison, ST6Gal1 remodeling of the G0 glycoform did not reach the level of completion found with ST3Gal3 remodeling. Figure 115A-115D and Figure 116A-116C show the results obtained from CE and HPLC analysis, respectively. No S2G2 glycoforms were seen in any of the glycoremodeled samples. However, all of the G2 glycoforms were converted into S1-G2. Analysis from MALDI-MS also supports these data (Figures 117A-117C).

**Stability of remodeled glycans of Cri-IgG1.** Lastly, the stability of the Cri-IgG1 glycans remodeled by exoglycosidase treatment and glycosylation was investigated. Each glycoremodeled Cri-IgG1 antibody was stored at 4°C, and was checked by SDS-PAGE for degradation at two weeks after remodeling. As shown in Figure 118A-118E, the remodeled DEAE and SPA antibodies both retained a molecular weight of about 150 kDa, indicating little to no degradation, regardless of the kind of glycoremodeling performed. The Fc Cri-IgG1 antibody retained a molecular weight of about 38 kDa, also indicating little to no degradation, regardless of the kind of remodeling performed.

Effector Function Bioassay of Remodeled Cri-IgG1 antibodies. The effector function bioassay was derived from the procedure of Mimura et al. (2000, Molecular Immunology 37:697-706). The IC<sub>50</sub> of the glycoforms of Cri-IgG1 antibody was determined by inhibition of the superoxide response of U937 cells elicited by red blood cells sensitized with native anti-NIP antibody.

Monocytic U937 cells were cultured in the presence of 1000 units/mL interferon gamma for 2 days to induce the differentiation of the cells and their capacity to generate superoxide. The cells were then washed and resuspended at  $2 \times 10^6$  cells/mL in Hanks balanced salt solution without phenol red and containing 20 mM HEPES pH 7.4 and 0.15 mM BSA. The red blood cells were sensitized with anti-NIP (5-iodo-4-hydroxy-3-nitrophenacetyl) antibody, in the absence or presence of the various glycoforms of Cri-IgG1 antibody, with incubation at 37°C for 30 minutes. The cells were then washed three times with PBS and resuspended at  $2.5 \times 10^7$  cells/mL in HBSS-BSA. The U937 cells ( $100 \mu\text{l}$ ,  $2 \times 10^6$  cells/mL) were added to plastic tubes and lucigenin ( $20 \mu\text{l}$ , 2.5 mM) was added to the tubes. The tubes were warmed in a 37°C water bath for 5 minutes. The sensitized red blood

cells (80  $\mu$ l,  $2.5 \times 10^7$  /mL) were then added to the tubes. Superoxide anion production was measured by lucigenin-enhanced chemiluminescence at 37°C over a 30 minute period using a Berthold LV953 luminometer (Berthold Australia Pty Ltd, Bundoora, Australia).

The G0 and M3N2 glycoforms Cri-IgG1 antibody had relative inhibitory values of 92% and 85%, respectively, as compared with the native antibody. However, the native CRI-IgG1 antibody lacked core fucose. Shields et al. (2002, J. Biol. Chem. 277:26733-26740) suggests that the lack of core fucose will improve inhibitory values 10 fold. Based on these results, it is anticipated that inhibitory values of the galactosylated-bisecting-G0 glycoform will be greater than the bisecting-G0 glycoform, which in turn will be much greater than the G2 glycoform, which in turn will be approximately equal to the disialylated-G2 glycoform and the monosialylated-G2 glycoform, which in turn will be greater than the native antibody glycoform, which in turn will be greater than the G0 glycoform, which in turn will be greater than the M3N2 glycoform.

## Complement Receptor-1

### 9. Sialylation and Fucosylation of TP10

This example sets forth the preparation of TP10 with sialyl Lewis X moieties and analysis of enhanced biological activity.

Interrupting blood flow to the brain, even for a short time, can trigger inflammatory events within the cerebral microvasculature that can exacerbate cerebral tissue damage. The tissue damage that accrues is amplified by activation of both inflammation and coagulation cascades. In a murine model of stroke, increased expression of P-selectin and ICAM-1 promotes leukocyte recruitment. sCR1 is recombinant form of the extracellular domain of Complement Receptor-1 (CR-1). sCR-1 is a potent inhibitor of complement activation. sCR1sLe<sup>X</sup> (CD20) is an alternately glycosylated form of sCR1 that is alternately glycosylated to display sialylated Lewis<sup>X</sup> antigen. Previously, sCR-1sLe<sup>X</sup> that was expressed and glycosylated *in vivo* in engineered Lec11 CHO cells was found to correctly localize to ischemic cerebral microvessels and C1q-expressing neurons, thus inhibiting neutrophil and platelet accumulation and reducing cerebral infarct volumes (Huang et al., 1999, Science 285:595-599). In the present example, sCR1sLe<sup>X</sup> which was prepared *in vitro*



by remodeling of glycans, exhibited enhanced biological activity similar to that of sCRsLe<sup>x</sup> glycosylated *in vivo*.

The TP10 peptide was expressed in DUK B11 CHO cells. This CHO cell line produces the TP10 peptide with the typical CHO cell glycosylation, with many but not all glycans capped with sialic acid.

**Sialylation of 66 mg of TP10.** TP10 (2.5 mg/mL), CMPSA (5 mM), and ST3Gal3 (0.1 U/mL) were incubated at 32°C in 50 mM Tris, 0.15M NaCl, 0.05% sodium azide, pH 7.2 for 48 hours. Radiolabelled CMP sialic acid was added to a small aliquot to monitor incorporation. TP10 was separated from nucleotide sugar by SEC HPLC. Samples analyzed at 24 hours and 48 hours demonstrated that the reaction was completed after 24 hours. The reaction mixture was then frozen. The reaction products were subjected to Fluorophore Assisted Carbohydrate Electrophoresis (FACE®; Glyko, Inc, Novato CA) analysis (Figure 119).

**Pharmacokinetic studies.** Rats were purchased with a jugular vein cannula. 10 mg/kg of either the pre-sialylation or post-sialylation TP10 peptide was given by tail vein injection to three rats for each treatment (n=3). Fourteen blood samples were taken from 0 to 50 hours. The concentration in the blood of post-sialylation TP10 peptide was higher than that of pre-sialylation TP10 at every time point past 0 hour (Figure 120). Sialic acid addition doubled the area under the plasma concentration-time curve (AUC) of the pharmacokinetic curve as compared to the starting material (Figure 121).

**Fucosylation of sialylated TP10.** 10 mL (25 mg TP10) of the above sialylation mix was thawed, and GDP-fucose was added to 5 mM, MnCl<sub>2</sub> to 5 mM, and FTVI (fucosyltransferase VI) to 0.05 U/mL. The reaction was incubated at 32°C for 48 hours. The reaction products were subjected to Fluorophore Assisted Carbohydrate Electrophoresis (FACE®; Glyko, Inc, Novato CA) analysis (Figure 122). To a small aliquot, radiolabelled GDP-fucose was added to monitor incorporation. TP10 was separated from nucleotide sugar by SEC HPLC. Samples analyzed at 24 hours and 48 hours demonstrated that the reaction was completed at 24 hours. An *in vitro* assay measuring binding to E-selectin indicate that fucose addition can produce a biologically-active E-selectin ligand (Figure 123).

Enbrel™10. GlycoPEGylation of an antibody Enbrel™

This example sets forth the procedures to PEGylate the O-linked glycans of an antibody molecule. Here, Enbrel™ is used as an example, however one of skill in the art will appreciate that this procedure can be used with many antibody molecules.

**Preparation of Enbrel™-SA-PEG (10 kDa).** Enbrel™ (TNF-receptor-IgG<sub>1</sub>-chimera), either with the O-linked glycans sialylated prior to PEGylation or not, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 5 mM UDP-galactose and 0.1 U/mL of galactosyltransferase at 32°C for 2 days to cap the undergalactosylated glycans with galactose. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G2000SW analytical column in methanol and water. The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the solution is incubated with 1 mM CMP-sialic acid-linker-PEG (10 kDa) and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid-linker-PEG, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

Erythropoietin (EPO)11. Addition of GlcNAc to EPO

This example sets forth the addition of a GlcNAc residue on to a tri-mannosyl core.

**Addition of GlcNAc to EPO.** EPO was expressed in SF-9 insect cells and purified (Protein Sciences, Meriden, CT). A 100% conversion from the tri-mannosyl glycoform of

Epo to the "tri-mannosyl core + 2 GlcNAc" (Peak 1, P1 in Figure 124) was achieved in 24 hours of incubation at 32°C with 100mU/ml of GlcNAcT-I and 100mU/ml of GlcNAcT-II in the following reaction final concentrations:

100mM MES pH 6.5, or 100mM Tris pH 7.5

5 5mM UDP-GlcNAc

20mM MnCl<sub>2</sub>

100mU/ml GlcNAcT-I

100mU/ml GlcNAcT-II

10 1 mg/ml EPO (purified, expressed in Sf9 cells,  
purchased from Protein Sciences).

**Analysis of glycoforms.** This assay is a slight modification on K-R Anumula and ST Dhume, Glycobiology 8 (1998) 685-69. N-glycanase (PNGase) released N-glycans were reductively labeled with anthranilic acid. The reductively-aminated N-glycans were injected onto a Shodex Asahipak NH2P-50 4D amino column (4.6 mm x 150 mm). Two solvents  
15 were used for the separation: A) 5% (v/v) acetic acid, 1% tetrahydrofuran, and 3% triethylamine in water, and B) 2% acetic acid and 1% tetrahydrofuran in acetonitrile. The column was then eluted isocratically with 70% B for 2.5 minutes, followed by a linear gradient over a period of 97.5 minutes going from 70 to 5% B and a final isocratic elution  
20 with 5% B for 15 minutes. Eluted peaks were detected using fluorescence detection with an excitation of 230 nm and emission wavelength of 420 nm.

Under these conditions, the trimannosyl core had a retention time of 22.3 minutes, and the product of the GnT reaction has a retention time of 30 minutes. The starting material was exclusively trimannosyl core with core GlcNAc (Figure 124).

## 25 12. Preparation of EPO with multi-antennary complex glycans

This example sets forth the preparation of PEGylated, biantennary EPO, and triantennary, sialylated EPO from insect cell expressed EPO.

Recombinant human erythropoietin (rhEPO) from the baculovirus/Sf9 expression system (Protein Sciences Corp., Meriden, CT) was subjected to glycan analysis and the  
30 resulting glycans were shown to be primarily trimannosyl core with core fucose, with a small percentage of glycans also having a single GlcNAc.

**Addition of N-acetylglucosamine with GnT-I and GnT-II.** Two lots of rhEPO (1 mg/mL) were incubated with GnT-I and GnT-II, 5 mM UDP-glcNAc, 20 mM MnCl<sub>2</sub>, and 0.02% sodium azide in 100 mM MES pH 6.5 at 32°C for 24hr. Lot A contained 20 mg of EPO, and 100 mU/mL GnT-I and 60 mU/mL GnT-II. Lot B contained 41 mg of EPO, and 41 mU/mL GnT-I + 50 mU/mL GnT-II. After the reaction, the sample was desalted by gel filtration (PD10 columns, Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

**EPO glycans analyzed by 2-AA HPLC profiling.** This assay is a slight modification on Anumula and Dhume, *Glycobiology* 8 (1998) 685-69. Reductively-aminated N-glycans were injected onto a Shodex Asahipak NH2P-50 4D amino column (4.6 mm x 150 mm). Two solvents were used for the separation, A) 5% (v/v) acetic acid, 1% tetrahydrofuran, and 3% triethylamine in water and B) 2% acetic acid and 1% tetrahydrofuran in acetonitrile. The column was then eluted isocratically with 70% B for 2.5 min, followed by a linear gradient over a period of 100 min going from 70 to 5% B, and a final isocratic elution with 5% B for 20 min. Eluted peaks were detected using fluorescence detection with an excitation of 230 nm and emission wavelength of 420 nm. Non-sialylated N-linked glycans fall in the LC range of 23-34 min, monosialylated from 34-42 min, disialylated from 42-52 min, trisialylated from 55-65 min and tetrasialylated from 68 – 78 min.

Glycan profiling by 2AA HPLC revealed that lot A was 92% converted to a biantennary structure with two GlcNAcs (the balance having a single GlcNAc. Lot B showed 97% conversion to the desired product (Figure 125A and 125B ).

**Introducing a third antennary branch with GnT-V.** EPO (1 mg/mL of lot B) from the product of the GnT-I and GnT-II reactions, after desalting on PD-10 columns and subsequent concentration, was incubated with 10 mU/mL GnT-V and 5 mM UDP-GlcNAc in 100 mM MES pH 6.5 containing 5 mM MnCl<sub>2</sub> and 0.02% sodium azide at 32°C for 24 hrs. 2AA HPLC analysis demonstrated that the conversion occurred with 92% efficiency (Figure 126).

After desalting (PD-10) and concentration, galactose was added with rGalTI: EPO (1 mg/mL) was incubated with 0.1 U/mL GalTI, 5 mM UDP-galactose, 5 mM MnCl<sub>2</sub> at 32°C for 24 hrs.

**MALDI analysis of reductively-aminated N-glycans from EPO.** A small aliquot of the PNGase released N-glycans from EPO that had been reductively labeled with

anthranilic acid was dialyzed for 45 min on an MF-Millipore membrane filter (0.025  $\mu$ m pore, 47 mm dia), which was floating on water. The dialyzed aliquot was dried in a speedvac, redissolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (10 g/L) dissolved in water/acetonitrile (50:50). The mixture was dried onto the target and analyzed using an Applied Biosystems DE-Pro MALDI-TOF mass spectrometer operated in the linear/negative-ion mode. Oligosaccharides were assigned based on the observed mass-to-charge ratio and literature precedence.

Analysis of released glycans by MALDI showed that galactose was added quantitatively to all available sites (Figure 127). Galactosylated EPO from above was then purified by gel filtration on a Superdex 1.6/60 column in 50 mM Tris, 0.15M NaCl, pH 6.

**Sialylation.** After concentration and desalting (PD-10), 10 mg galactosylated EPO (1 mg/mL) was incubated with ST3Gal3 (0.05 U/mL), and CMP-SA (3 mM) in 50 mM Tris, 150 mM NaCl, pH 7.2 containing 0.02% sodium azide. A separate aliquot contained radiolabelled CMP-SA. The resulting incorporated label and free label was separated by isocratic size exclusion chromatography/HPLC at 0.5mL/min in 45% MeOH, 0.1%TFA (7.8mm x 30 cm column, particle size 5  $\mu$ m, TSK G2000SW<sub>XL</sub>, Toso Haas, Ansys Technologies, Lake Forest, CA). Using this procedure, 12% of the counts were incorporated (360 micromolar, at 33 micromolar EPO, or about 10.9 moles/mole). Theoretical (3 N-linked sites, tri-antennary) is about 9 moles/mole incorporation. These correspond within the limits of the method. In an identical reaction with ST6Gal1 instead of ST3Gal3, 5.7% of the radiolabel was incorporated into the galactosylated EPO, or about 48% compared with ST3Gal3.

### 13. GlycoPEGylation of EPO produced in insect cells

This example sets forth the preparation of PEGylated biantennary EPO from insect cell expressed EPO.

Recombinant human erythropoietin (rhEPO) from the baculovirus/Sf9 expression system (Protein Sciences Corp., Meriden, CT) was subjected to glycan analysis and the resulting glycans were shown to be primarily trimannosyl core with core fucose, with a small percentage of glycans also having a single GlcNAc (Figure 128).

**Addition of N-acetylglucosamine with GnT-I and GnT-II.** Two lots of rhEPO (1 mg/mL) were incubated with GnT-I and GnT-II, 5 mM UDP-glcNAc, 20 mM MnCl<sub>2</sub>, and 0.02% sodium azide in 100 mM MES pH 6.5 at 32°C for 24hr. Lot A contained 20 mg of EPO, and 100 mU/mL GnT-I and 60 mU/mL GnT-II. Lot B contained 41 mg of EPO, and 41 mU/mL GnT-I + 50 mU/mL GnT-II. After the reaction, the sample was desalted by gel filtration (PD10 columns, Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Glycan profiling by 2AA HPLC revealed that lot A was 92% converted to a biantennary structure with two GlcNAcs (the balance having a single glcNAc. Lot B showed 97% conversion to the desired product (Figure 125A and 125B ).

**Galactosylation of EPO lot A.** EPO (~16 mgs of lot A) was treated with GnT-II to complete the addition of GlcNAc. The reaction was carried out in 50 mM Tris pH 7.2 containing 150 mM NaCl, EPO mg/ml, 1 mM UDP-GlcNAc, 5 mM MnCl<sub>2</sub>, 0.02% sodium azide and 0.02 U/ml GnT-II at 32 °C for 4 hrs. Then galactosylation of EPO was done by adding UDP-galactose to 3 mM and GalT1 to 0.5 U/ml and the incubation continued at 32° C for 48 hrs.

Galactosylated EPO was then purified by gel filtration on a Superdex75 1.6/60 column in 50 mM Tris, 0.15M NaCl, pH 6. The EPO containing peak was then analyzed by 2AA HPLC. Based on the HPLC data ~85% of the glycans contains two galactose and ~15% of the glycans did not have any galactose after galactosylation reaction.

**Sialylation of galactosylated EPO.** Sialylation of galactosylated EPO was carried out in 100 mM Tris pH containing 150 mM NaCl, 0.5 mg/ml EPO, 200 mU/ml of ST3Gal3 and either 0.5 mM CMP-SA or CMP-SA-PEG (1 kDa) or CMP-SA-PEG (10 kDa) for 48 hrs at 32 °C. Almost all of the glycans that have two galactose residues were fully sialylated (2 sialic acids / glycan) after sialylation reaction with CMP-SA. MALDI-TOF analysis confirmed the HPLC data.

**PEGylation of galactosylated EPO.** For PEGylation reactions using CMP-SA-PEG (1 kDa) and CMP-SA-PEG (10 kDa), an aliquot of the reaction mixture was analyzed by SDS-PAGE (Figure 129). The molecular weight of the EPO peptide increased with the addition of each sugar, and increased more dramatically in molecular weight after the PEGylation reactions.

***In vitro* bioassay of EPO.** *In vitro* EPO bioassay (adapted from Hammerling et al, 1996, J. Pharm. Biomed. Anal. 14: 1455-1469) is based on the responsiveness of the TF-1 cell line to multiple levels of EPO. TF-1 cells provide a good system for investigating the proliferation and differentiation of myeloid progenitor cells. This cell line was established by  
5 T. Kitamura et al. in October 1987 from a heparinized bone marrow aspiration sample from a 35 year old Japanese male with severe pancytopenia. These cells are completely dependent on Interleukin 3 or Granulocyte-macrophage colony-stimulating factor (GM-CSF).

The TF-1 cell line (ATCC, Cat. No. CRL-2003) was grown in RPMI + FBS 10% + GM-CSF (12 ng/ml) and incubated at 37°C 5% CO<sub>2</sub>. The cells were in suspension at a  
10 concentration of 5000 cells/ml of media, and 200 µl were dispensed in a 96 well plate. The cells were incubated with various concentrations of EPO (0.1 µg/ml to 10 µg/ml) for 48 hours. A MTT Viability Assay was then done by adding 25 µl of MTT at 5 mg/ml (SIGMA M5655), incubating the plate at 37°C for 20 min to 4 hours, adding 100 µl of  
15 isopropanol/HCl solution (100 ml isopropanol + 333 µl HCl 6N), reading the OD at 570 nm, and 630nm or 690nm, and subtracting the readings at 630 nm or 690 nm from the readings at 570 nm.

Figure 130 contains the results when sialylated EPO, and EPO glycoPEGylated with 1 kDa or 10 kDa PEG was subjected to an *in vitro* EPO bioactivity test. The EPO glycoPEGylated with 1kDa PEG had almost the same activity as the unglycoPEGylated EPO  
20 when both were at a concentration of approximately 5 µg/ml. The EPO glycoPEGylated with 10 kDa PEG had approximately half the activity of the unglycoPEGylated EPO when both were at a concentration of approximately 5 µg/ml.

#### 14. GlycoPEGylation of O-Linked Glycans of EPO produced in CHO Cells

**Preparation of O-linked EPO-SA-PEG (10 kDa).** Asialo-EPO, originally produced  
25 in CHO cells, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 5 mM CMP-SA and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid onto the N-linked glycans, a small aliquot of the reaction had CMP-SA-<sup>14</sup>C added; the peptide is separated by gel filtration on a Toso  
30 Haas G2000SW analytical column using methanol, water and the product detected using a

radiation detector. When the reaction is complete, the solution is concentrated using a Centricon-20 filter. The remaining solution is buffer exchanged with 0.05 M Tris (pH 7.2), 0.15 M NaCl, 0.05% NaN<sub>3</sub> to a final volume of 7.2 mL until the CMP-SA could no longer be detected. The retentate is then resuspended in 0.05 M Tris (pH 7.2), 0.15 M NaCl, 0.05% NaN<sub>3</sub> at 2.5 mg/mL protein. The solution is incubated with 1 mM CMP-SA-PEG (10 kDa) and ST3Gal1, to glycosylate the O-linked site, at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction is separated by gel filtration using a Toso Haas TSK-gel-3000 analytical column eluting with PBS pH 7.0 and analyzing by UV detection. When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-gel-3000 preparative column using PBS buffer (pH 7.0) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

#### 15                    15. EPO-Transferrin

This example sets forth the procedures for the glycoconjugation of proteins to O-linked glycans, and in particular, transferrin is glycoconjugated to EPO. The sialic acid residue is removed from O-linked glycan of EPO, and EPO-SA-linker-SA-CMP is prepared. EPO-SA-linker-SA-CMP is glycoconjugated to asialotransferrin with ST3Gal3.

20                    **Preparation of O-linked asialo-EPO.** EPO (erythropoietin) produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is incubated with 300 mU/mL sialidase (*Vibrio cholera*)-agarose conjugate for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The supernatant is concentrated to a EPO concentration of about 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 5 mM CMP-sialic acid and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid, a small aliquot of the reaction had CMP-SA-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). When the reaction is complete, the reaction mixture is purified using a Toso



Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

5       **Preparation of EPO-SA-linker-SA-CMP.** The O-linked asialo-EPO 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-linker-SA-CMP and 0.1 U/mL of ST3Gal1 at 32°C for 2 days. To monitor the incorporation of sialic acid-linker-SA-CMP, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1).

10       After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

15       **Preparation of Transferrin-SA-Linker-SA-EPO.** EPO-SA-Linker-SA-CMP from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL asialo-transferrin and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of transferrin, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1) and the  
20       product detected by UV absorption. When the reaction is complete, the solution is incubated with 5 mM CMP-SA and 0.1 U/mL of ST3Gal3 (to cap any unreacted transferrin glycans) at 32°C for 2 days. The reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures  
25       and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

#### 16. EPO-GDNF

30       This example sets forth the procedures for the glycoconjugation of proteins, and in particular, the preparation of EPO-SA-Linker-SA-GDNF.

**Preparation of EPO-SA-Linker-SA-GDNF.** EPO-SA-Linker-SA-CMP from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL GDNF (produced in NSO) and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of GDNF, the peptide is separated by gel  
5 filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1) and the product detected by UV absorption. When the reaction is complete, the solution is incubated with 5 mM CMP-SA and 0.1 U/mL of ST3Gal3 (to cap any unreacted GDNF glycans) at 32°C for 2 days. The reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product  
10 of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

#### 17. Mono-antennary GlycoPEGylation of EPO

15 This example sets forth the procedure for the preparation of glycoPEGylated mono-antennary erythropoietin (EPO), and its bioactivity *in vitro* and *in vivo*.

When EPO (GenBank Accession No. P01588) is expressed in CHO cells, N-linked glycans are formed at amino acid residues 24, 38 and 83, and an O-linked glycan is formed at amino acid residue 126 (Fig. 131; Lai et al., 1986, J. Biol. Chem. 261:3116-3121). The  
20 bioactivity of this glycoprotein is directly correlated with the level of NeuAc content. Increased sialic acid decreases the binding of EPO to its receptor *in vitro*; however increased sialic acid increases the bioactivity of EPO *in vivo*. The O-linked glycan has no impact on the *in vitro* or *in vivo* activity of EPO, or the pharmacokinetics of the molecule (Wasley et al., 1991, Blood 77:2624-2632).

25 When EPO is expressed in insect cells, such as is accomplished using a baculovirus/Sf9 expression system (see also, Wojchowshi et al., 1987, Biochem. Biophys. Acta 910:224-232; Quelle et al., 1989, Blood 74:652-657), N-linked glycans are formed at amino acid residues 24, 38 and 83, but an O-linked glycan is not formed at amino acid residue 126 (Fig. 132). This is because the insect cell does not have a glycosyl transferase  
30 that recognizes the amino acid sequence around amino acid residue 126 of EPO. The majority of the N-linked glycans are composed of GlcNAc<sub>2</sub>Man<sub>3</sub>Fuc. In the present example,

EPO expressed in insect cells was remodeled with high efficiency to achieve the complex glycan SA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>FucGlcNAc<sub>2</sub> by contacting the protein with, in series, GnT1,2, GalT-1, and ST in the presence of the appropriate donor molecules. These enzymatic reactions were performed on insect cell expressed EPO using reaction conditions disclosed herein, to yield the complex glycans herein with 92% total efficiency (Table 21). Optionally, O-linked glycans can also be added (O'Connell and Tabak, 1993, J. Dent. Res. 72:1554-1558; Wang et al., 1993, J. Biol. Chem. 268:22979-22983).

**Table 21.** Percent of each species of glycan structure in the population of glycan structures on insect cell expressed EPO ("starting material"), and on EPO after each sequential enzymatic remodeling step.

Glycan	Starting Material	After GnT-I, II	After GalT-I	After ST
	0.5%			
	98.0%			
	1.0%	0.5%	0.5%	
	0.5%	99.5%	4%	2%
			95.9%	5%
				92.0%

= fucose, 
 = GlcNAc, 
 = mannose, 
 = galactose, 
 = N-acetylneuraminic acid

Also in the present example, EPO expressed in insect cells was remodeled to form mono-antennary, bi-antennary and tri-antennary glycans, which were subsequently glycoPEGylated with 1 kDa, 10 kDa and 20 kDa PEG molecules using procedures described elsewhere herein. The molecular weights of these EPO forms were determined, and were compared to Epoetin™ having 3 N-linked glycans, and NESP (Aranesp™) having 5 N-linked glycans (Fig. 133). Examples of the preparation of bi- and tri-antennary glycan structures are given in Example 7, herein.

EPO having monoantennary PEGylated glycan structures is prepared by expressing EPO peptide in insect cells, then contacting the EPO peptide with GnTI only (or alternatively GnTII only) in the presence of a GlcNAc donor. The EPO peptide is then contacted with GalT-I in the presence of a galactose donor. The EPO peptide is then contacted with ST in the presence of SA-PEG donor molecules (Fig. 134A) to generate an EPO peptide having three N-linked mono-antennary PEGylated glycan structures (Fig. 134B).

The *in vitro* bioactivity of EPO-SA and EPO-SA-PEG generated from insect cell expressed EPO was accessed by measuring the ability of the molecule to stimulate the proliferation of TF-1 erythroleukemia cells. Tri-antennary EPO-SA-PEG 1 kDa exhibited almost all of the bioactivity of tri-antennary EPO-SA, and di-antennary EPO-SA-PEG 10 kDa exhibited almost all of the bioactivity of di-antennary EPO-SA over a range of EPO concentrations (Fig. 135). Remodeled and glycoPEGylated EPO generated in insect cells exhibited up to 94% of the *in vitro* bioactivity of Epogen™, which is EPO expressed in CHO cells without further glycan remodeling or PEGylation (Table 22).

**Table 22.** *In vitro* activity of the EPO constructs as compared with Epogen™ at 2 µg/ml protein and 48 hr.

Compound (2 µg/ml protein)	Activity ( percent of Epogen™)
Biantennary-SA	146
Biantennary-SA-PEG 1K	94
Biantennary-SA-PEG 10K	75
Triantennary-SA 2,3 <sup>1</sup>	42
Triantennary-SA-PEG 1K	48
Triantennary-SA-PEG 10K	34

<sup>1</sup> The triantennary -SA 2,3 construct has the SA molecule bonded in a 2,3 linkage.

The *in vivo* pharmacokinetics of glycoPEGylated and non-glycoPEGylated EPO was determined. GlycoPEGylated and non-glycoPEGylated [<sup>125</sup>I]-labeled EPO was bolus injected into rats and the pharmacokinetics of the molecules were determined. As compared with bi-antennary EPO, the AUC of bi-antennary EPO-PEG 1 kDa was 1.8 times greater, and the AUC of bi-antennary EPO-PEG 10 kDa was 11 times greater (Fig. 136). As compared with

bi-antennary EPO, the AUC of bi-antennary EPO-PEG 1 kDa was 1.6 times greater, and the AUC of bi-antennary EPO-PEG 10 kDa was 46 times greater (Fig. 136). Therefore, the pharmacokinetics of EPO was greatly improved by glycoPEGylation.

The *in vivo* bioactivity of glycoPEGylated and non-glycoPEGylated EPO was also determined by measuring the degree to which the EPO construct could stimulate reticulocytosis. Reticulocytosis is a measure of the rate of the maturation of red blood cell precursor cells into mature red blood cells (erythrocyte). Eight mice per treatment group were given a single subcutaneous injection of 10 µg protein/Kg, and the percent reticulocytes was measured at 96 hours (Fig. 137). Tri- and bi-antennary PEGylated EPO exhibited greater *in vivo* bioactivity than non-PEGylated EPO forms, including Epogen™.

Further determination of *in vivo* bioactivity of the EPO constructs was assessed by measuring the hematocrit (the percent of whole blood that is comprised of red blood cells) of CD-1 female mice 15 days after intraperitoneal injection three times per week with 2.5 µg peptide/kg body weight of the EPO construct. The hematocrit increment increased with the size of the EPO form, with the 82.7 kDa mono-antennary EPO-PEG 20 kDa having a slightly greater activity than the 35.6 kDa NESP (Aranesp™) and about two times the bioactivity of 28.5 kDa Epogen™ (Fig. 138).

This example illustrates that the generation of a longer-acting glycoPEGylated EPO is feasible. The pharmacokinetic profile of glycoPEGylated EPO can be customized by altering the number of glycoPEGylation sites and the size of the PEG molecule added to alter the half-life of the peptide in the bloodstream. Finally, glycoPEGylated EPO retains both *in vitro* and *in vivo* bioactivity.

#### 18. Preparation and Bioactivity of Sialylated and PEGylated Mono-, Bi- and Tri-Antennary EPO

This example illustrates the production of glycoPEGylated EPO, in particular PEGylated EPO having mono-antennary and bi-antennary glycans with PEG linked thereto. The following EPO variants were produced: mono-antennary PEG (1 kDa), PEG (10 kDa) and PEG (20 kDa); bi-antennary 2,3-sialic acid (SA), bi-antennary SA-PEG (1 kDa), bi-antennary SA-PEG (10 kDa); tri-antennary 2,3-SA and tri-antennary 2,6-SA capped with 2,3-SA.

Recombinant erythropoietin (rEPO) expressed in insect cells was obtained from Protein Sciences (Lot # 060302, Meridan CT). The glycan composition of this batch of EPO had approximately 98% trimannosyl core structure. Figure 139A depicts the HPLC analysis of the released glycans from this EPO, with peak "P2" representing the trimannosyl core glycan. Figure 139B shows the MALDI analysis of the released glycans with the structures of the released glycans beside the peak they represent.

#### **Mono-antennary branching**

Several steps were performed to produce the mono-antennary branched structure. In brief, the first step was a GnT-I/GalT-1 reaction followed by purification using Superdex-75 chromatography. This reaction adds a GlcNAc moiety to one branch of the tri-mannosyl core, and a galactose moiety onto the GlcNAc moiety. Branching was extended with the ST3Gal3 reaction to add the SA-PEG (10 kDa) moiety or the SA-PEG (20 kDa) moiety onto the terminal galactose moiety. The final purification was accomplished using Superdex-200 chromatography (Amersham Biosciences, Arlington Heights, IL).

**GnT-I/GalT-1 Reaction.** The GnT-I and GalT-1 reactions were combined and incubated at 32°C for 36 hours. The reaction contained 1 mg/mL EPO, 100 mM Tris-Cl pH 7.2, 150 mM NaCl, 5 mM MnCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 3 mM UDP-GlcNAc, 50 mU/mg GnT-I, 3 mM UDP-Gal, and 200 mU/mg GalT-1. Figure 140 depicts the MALDI analysis of glycans released from EPO after the GnT-I/GalT-1 reaction. Glycan analysis showed approximately 90% of the glycans had the desired mono-antennary branched structure with a terminal galactose moiety.

**Superdex 75 Purification.** After the GnT-I/GalT1 reaction, EPO was purified from the enzyme protein contaminants and nucleotide sugars using a 1.6 cm x 60 cm Superdex-75 gel filtration chromatography (Amersham Biosciences, Arlington Heights, IL) in PBS containing 0.02% Tween 20 (Sigma-Aldrich Corp., St. Louis, MO).

**ST3Gal3 Reaction.** The ST3Gal3 PEGylation reaction was incubated at 32°C for 24 hours. The reaction contained 1 mg/mL EPO, 100 mM Tris-Cl pH 7.2, 150 mM NaCl, 0.02% NaN<sub>3</sub>, 200 mU/mg ST3Gal3, and 0.5 mM CMP-SA-PEG (10 kDa) or 0.5 mM CMP-SA-PEG (20 kDa). Figure 141 depicts the SDS-PAGE analysis of EPO after this reaction. The corresponding molecular weights of the protein bands indicate that the EPO glycans formed by the GnT-I/GalT-1 reaction were completely sialylated with the PEG derivative.

**Superdex 200 Purification.** EPO then was purified from the contaminants of the ST3Gal3 reaction by a 1.6 cm x 60 cm Superdex-200 gel filtration chromatography (Amersham Biosciences, Arlington Heights, IL) in PBS containing 0.02% Tween-20.

**TF-1 Cell *In Vitro* Bioassay of Mono-antennary PEGylated EPO.** The TF-1 cell line is used to assess the activity of EPO *in vitro*. The TF-1 cells line is a myeloid progenitor cell line available from the American Type Culture Collection (Catalogue No. CRL-2003, Rockville, MD). The cell line is completely dependant on Interleukin-3 or Granulocyte-Macrophage Colony-Stimulating Factor for viability. TF-1 cells provide a good system for investigating the effect of EPO on proliferation and differentiation.

The TF-1 cells were grown in RPMI with 10% FBS and 12 ng/ml GM-CSF at 37°C in 5% CO<sub>2</sub>. The cells were suspended at a concentration of 10,000 cells/ml of media. 200 µl aliquots of cells were dispensed into a 96-well plate. The cells were incubated with 0.1 to 10 µg/ml EPO for 48 hrs.

The MTT viability assay was then performed by first adding 25 µl of 5 µg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, or thiazolyl blue; Sigma Chemical Co., St. Louis, Mo., Catalogue No. M5655). The plate was incubated for 4 hrs at 37°C. 100 µl of isopropanol/HCl solution (100 ml isopropanol and 333 µl HCl 6N) was added. The absorbency of the plates was read at 570 nm and either 630 or 690 nm, and the reading at either 630 nm or 690 nm was subtracted for the reading at 570 nm.

Figure 142 depicts the results of the bioassay of EPO activity after PEGylation of it mono-antennary glycans. In this bioassay, the mono-antennary PEGylated EPO is much less active than a non-PEGylated EPO (Epogen).

***In Vivo* Bioassay of Mono-antennary PEGylated EPO – Examination of Hematological Parameters.**

The effect of EPO-SA-PEG (10 kDa) and EPO-SA-PEG (20 kDa), the preparation of which compounds is described in detail above, on hematological parameters of male Sprague Dawley rats was examined. Sprague Dawley rats are a well-studied experimental model and techniques for the use and care of such animals are well-known to those skilled in the art. Therefore, Sprague Dawley rats provide a good system for studying the clinical and

physiological effects of EPO-SA-PEG (10 kDa) and EPO-SA-PEG (20 kDa) according to the present invention.

**Preparation, Care, and Handling of Test Subjects.** *In vivo* testing in accordance with the methods and compositions of the present invention was conducted at TherImmune Research Corporation (Gaithersburg, MD). Test subjects for the *in vivo* studies included 170 male Sprague Dawley rats, obtained from Harlan (Harlan Sprague Dawley, Inc., Indianapolis, IN). Subjects were all at least 300 grams in weight and at least 70 days old at the time of shipment. Subjects were cared for according to TherImmune's Institutional Animal Care and Use Committee, in accordance with provisions of the USDA Animal Welfare Act, the PHS Policy on Humane Care and Use of Laboratory Animals, and the U.S. Interagency Research Animal Committee Principles for the Utilization and Care of Research Animals. The study was conducted in accordance with the FDA Good Laboratory Practice Standards, 21 CFR Part 58. Subjects were housed individually in polycarbonate cages, under controlled environmental conditions, and were provided fresh food weekly, *ad libitum*, and tap water was made available to test subjects *ad libitum* via either an automatic watering system or water bottles.

Subjects were randomly assigned to groups for testing, and the minimum number of animals needed to give biologically meaningful data were used. Subjects were dosed via subcutaneous injection, and dosing frequency and quantity is indicated in in Table 25, and were observed prior to dose administration, and at least twice daily for signs of toxicity, moribundity and mortality. Body weights were measured and physical examinations were conducted on subjects pre- and post-study, as well as on a weekly basis during the study.

For hematology studies, a 0.25 ml ( $\pm 15\%$ ) sample of blood was collected from the jugular vein of all surviving subjects prior to the first dose on study day one, as well as on study days 3, 5, 8, 10, 12, 15, 17, 19, 22, 24, 26 and 29. Hematological analysis of the blood of the test subjects included examination of cellular morphology, erythrocyte count, hematocrit, hemoglobin, leukocyte, leukocyte differential, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume, mean platelet volume, platelet count, and reticulocytes.



Table 25. Summary of test subject grouping and dosage.

Group	Treatment	Dose (ug/kg)	Dose Volume (ml/kg)	Concentration (ug/ml)	Dose Frequency	Number of Animals
1	Vehicle	-	0.33	-	Every 3 days <sup>1</sup>	10
2	P113	2.5	0.33	7.5	Every 3 days <sup>1</sup>	5
3	P113	6.0	0.33	18	Every 3 days <sup>1</sup>	5
4	P140	2.5	0.33	7.5	Every 3 days <sup>1</sup>	5
5	P140	6.0	0.33	18	Every 3 days <sup>1</sup>	5
6	P138	2.5	0.33	7.5	Every 3 days <sup>1</sup>	5
7	P138	6.0	0.33	18	Every 3 days <sup>1</sup>	5
8	P139	2.5	0.33	7.5	Every 3 days <sup>1</sup>	5
9	P139	6.0	0.33	18	Every 3 days <sup>1</sup>	5
10	Vehicle	-	0.33	-	Every 5 days <sup>2</sup>	10
11	P140	4	0.33	12	Every 5 days <sup>2</sup>	5
12	P140	12	0.33	36	Every 5 days <sup>2</sup>	5
13	P138	4	0.33	12	Every 5 days <sup>2</sup>	5
14	P138	12	0.33	36	Every 5 days <sup>2</sup>	5
15	P139	4	0.33	12	Every 5 days <sup>2</sup>	5
16	P139	12	0.33	36	Every 5 days <sup>2</sup>	5
17	Vehicle	-	0.33	-	Every 7 days <sup>3</sup>	10
18	P140	6	0.33	18	Every 7 days <sup>3</sup>	5
19	P140	25	0.33	75	Every 7 days <sup>3</sup>	5
20	P138	6	0.33	18	Every 7 days <sup>3</sup>	5
21	P138	25	0.33	75	Every 7 days <sup>3</sup>	5
22	P139	6	0.33	18	Every 7 days <sup>3</sup>	5
23	P139	25	0.33	75	Every 7 days <sup>3</sup>	5
24	Vehicle	-	0.33	-	Every 10 days <sup>4</sup>	10
25	P140	7.5	0.33	23	Every 10 days <sup>4</sup>	5
26	P140	50	0.33	150	Every 10 days <sup>4</sup>	5
27	P138	7.5	0.33	23	Every 10 days <sup>4</sup>	5
28	P138	50	0.33	150	Every 10 days <sup>4</sup>	5
29	P139	7.5	0.33	23	Every 10 days <sup>4</sup>	5
30	P139	50	0.33	150	Every 10 days <sup>4</sup>	5

5

Study days are indicated for each group of subjects as follows:

1 = SD 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28

2 = SD 1, 6, 11, 16, 21 and 27

3 = SD 1, 8, 15 and 22

4 = SD 1, 11 and 21

10

On study day 29, after clinical pathology blood collection, all surviving subjects were euthanized via carbon dioxide inhalation. Blood was collected from the abdominal aorta. Moribund subjects that were previously euthanized, as well as subjects found dead during the study, were subjected to full necropsy.

5        **Analysis of results and interpretation of data.** Quantitative results were analyzed using techniques known to those skilled in the relevant art. In particular, results were analyzed using the Kolmogorov-Smirnov test for normality, the Levene Median test for equal variance, and one-way Analysis of Variance (ANOVA). In instances where the normality or equal variance test failed, the analysis was continued using the non-parametric Kruskal-  
10       Wallis ANOVA on rank-transformed data. Parametric data was analyzed using the Dunnett's t-test to delineate which groups differed from the control. For non-parametric data, the Dunns test was used to delineate which groups differed from the control. A probability value of less than 0.05 (two-tailed) was used as the critical level of significance for all tests.

      Based on one analysis of the data from the first fifteen days of the study, it was shown  
15       that a single dose of 50 µg/kg of glycoPEGylated monoantennary EPO, either EPO-SA-PEG (10 kDa) or EPO-SA-PEG (20 kDa) according to the present invention, gives an extended rise in blood hemoglobin in rats, comparable to the effect of NESP given at the same dose (Figure 192). Not wishing to be bound by any particular theory, Figure 192 illustrates that EPO-SA-PEG (10 kDa) and EPO-SA-PEG (20 kDa) have unexpectedly enhanced biological  
20       properties.

#### **Bi-antennary Branching**

      Several reactions were performed to accomplish the bi-antennary branching of EPO. Briefly, the first reaction combined the GnT-I and GnT-II reactions to add GlcNAc moieties  
25       to two of the tri-mannosyl core branches. The second reaction, the GalT-1 reaction, adds a galactose moiety to each GlcNAc moieties. Superdex 75 chromatography (Amersham Biosciences, Arlington Heights, IL) was performed prior to the ST3Gal3 reaction. The bi-antennary branching was further extended with the ST3Gal3 reaction to add either a 2,3-SA, or SA-PEG (1 kDa), SA-PEG (10 kDa). Final purification was accomplished using Superdex  
30       200 chromatography (Amersham Biosciences, Arlington Heights, IL).

**GnT-I/GnT-II Reaction.** The GnT-I and GnT-II reactions were combined and incubated at 32°C for 48 hours. The reaction contained 1 mg/mL EPO, 100 mM MES pH 6.5, 150 mM NaCl, 20 mM MnCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 5 mM UDP-GlcNAc, 100 mU/mg GnT-I, 60 mU/mg GnT-II. The reaction achieved 92% completion of the addition of bi-antennary  
5 GlcNAc moieties, with 8% mono-antennary GlcNAc moieties. Figure 143A shows the HPLC analysis of the released glycans, where peak "P3" represents the bi-antennary GlcNAc glycan. Figure 143B depicts the MALDI analysis of the released glycans with the structures of the glycans indicated beside the peak that they represent.

In order to further the reaction, an additional 20 mU/mg of GnT-II was added along  
10 with 1 mM UDP-GlcNAc, 5 mM MnCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, and the mixture was incubated for 4 hours at 32°C. Greater than 99% of this reaction achieved completion of the bi-antennary GlcNAc glycan.

**GalT-1 Reaction.** The GalT-1 reaction was started immediately after the completion of the second GnT-II reaction. Enzyme and nucleotide sugar were added to the completed  
15 GnT-II reaction at concentrations of 0.5 U/mg GalT-1 and 3 mM UDP-Gal.

When the GalT-1 reaction was performed on a small scale, with about 100 µg EPO per reaction, approximately 95% of the reaction produced EPO with bi-antennary terminal galactose moiety. Figure 144A depicts the HPLC analysis of the released glycans where  
20 peak "P2" is the bi-antennary glycan with terminal galactose moieties (85% of the glycans), and peak "P1" is the bi-antennary glycan without the terminal galactose moieties (15% of the glycans).

The GalT-1 reaction was also performed on a large scale with about 16 mg of EPO per reaction. Figure 144B depicts the HPLC analysis of the release glycans from the large scale GalT-1 reaction, where peak "P2" is the bi-antennary glycan with terminal galactose  
25 moieties, and peak "P1" is the bi-antennary glycan without the terminal galactose moieties.

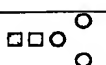
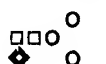

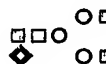
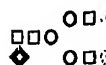
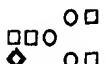
**Superdex 75 Purification.** EPO was then purified from the enzyme protein contaminants and nucleotide sugars using a 1.6 cm x 60 cm Superdex-75 gel filtration chromatography (Amersham Biosciences, Arlington Heights, IL) in PBS containing 0.02% Tween 20 after the GnT-1/GalT1 reaction. Figure 145 depicts the chromatogram of the  
30 Superdex 75 gel filtration, where peak 2 is EPO with bi-antennary glycans with terminal galactose moieties. Figure 146 shows SDS-PAGE analysis of the products of each

remodeling step indicating the increase in the molecular weight of EPO with each remodeling step.

**ST3Gal3 Reaction.** The ST3Gal3 reaction was incubated at 32°C for 24 hours. The reaction contained 0.5 mg/mL EPO, 100 mM Tris-Cl pH 7.2, 150 mM NaCl, 0.02% NaN<sub>3</sub>, 100 mU/mg ST3Gal3, and 0.5 mM CMP-SA, 0.5 mM CMP-SA-PEG (1 kDa), or 0.5 mM CMP-SA-PEG (10 kDa). Figure 147 shows the results of SDS-PAGE analysis of EPO before and after the ST3Gal3 reaction. Based on this SDS-PAGE analysis, bi-antennary EPO containing terminal Gal can no longer be visually detected after each ST3Gal3 reaction. All sialylated EPO variants show an increase in size compared to non-sialylated EPO at the start of the reaction.

**Superdex 200 Purification.** EPO was purified from the contaminants of the ST3Gal3 reactions by a 1.6 cm x 60 cm Superdex-200 gel filtration chromatography (Amersham Biosciences, Arlington Heights, IL) in PBS containing 0.02% Tween-20. Table 23 summarizes the distribution of glycan structures at each remodeling step.

**Table 23.** Summary of glycan structures on EPO after each remodeling step.

Glycan	Starting Material	After GnT-I and GnT-II	After 2nd GnT-II	After GalT-1	After ST
	0.5%				
	98.0%				
	1.0%	8.0%	0.5%	0.5%	0.5%
	0.5%	92.0%	99.5%	15.5%	15.5%
				84.0%	2.0%
					82.0%

Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose, open circles represent galactose.

### Tri-antennary Branching

Several reactions were performed to accomplish the tri-antennary branching of EPO. Briefly, the first reaction combined the GnT-I and GnT-II reactions to add a GlcNAc moiety to the two outer tri-mannosyl core branches of the glycan. The second reaction, GnT-V  
5 reaction, adds a second GlcNAc moiety to one of the two outer trimannosyl core branches so that there are now three GlcNAc moieties. The third reaction, GalT-1 reaction, adds a galactose moiety to each terminal GlcNAc moiety. The EPO products were then separated by Superdex 75 chromatography. The tri-antennary branching was further extended with the ST3Gal3 reaction to add either a 2,3-SA moiety or a 2,6-SA moiety, and capped with a 2,3-  
10 SA moiety. Final purification was accomplished using Superdex 75 chromatography.

**GnT-I/GnT-II Reaction.** The GnT-I and GnT-II reactions were combined and incubated at 32°C for 24 hours. The reaction contained 1 mg/mL EPO, 100 mM MES pH 6.5, 150 mM NaCl, 20 mM MnCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 5 mM UDP GlcNAc, 50 mU/mg GnT-I and 41 mU/mg GnT-II. The reaction achieved 97% completion of the addition of the bi-  
15 antennary GlcNAc moiety, with 3% tri-mannosyl core remaining. Figure 148 depicts the HPLC analysis of the glycans released from EPO after the GnT-I/GnT-II reaction.

**GnT-V Reaction.** The GnT-V reaction containing 100 mM MES pH 6.5, 5 mM UDP-GlcNAc, 5 mM MnCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 10 mU/mg GnT-V and 1 mg/mL EPO, was incubated at 32°C for 24 hours. This reaction adds a GlcNAc moiety to an outer mannose  
20 moiety already containing a GlcNAc moiety. Figure 149 depicts the HPLC analysis of the glycans released from EPO after the GnT-V reaction. Approximately 92% the glycans released from EPO were the desired product, tri-antennary branched EPO with terminal GlcNAc moieties, based on glycan and MALDI analysis. The remaining 8% of the glycans were bi-antennary branched structures containing terminal GlcNAc moieties.

**GalT-1 Reaction.** The GalT-1 reaction containing 100 mM Tris pH 7.2, 150 mM NaCl, 5 mM UDP Gal, 100 mU/mg GalT-1, 5 mM MnCl<sub>2</sub>, 0.02% NaN<sub>3</sub> and 1 mg/mL EPO was incubated at 32°C for 24 hours. Figure 150 depicts the HPLC analysis of the glycans released from EPO after this reaction. Glycan and MALDI analysis indicates that 97% of the released glycans had terminal galactose moieties on the tri-antennary branched structures.  
25  
30 The remaining 3% was a bi-antennary structure containing a terminal galactose.

**Superdex 75 Purification.** After the GnT-I/GalT1 reaction, EPO was purified from the enzyme protein contaminants and nucleotide sugars using a 1.6 cm x 60 cm Superdex-75 gel filtration chromatography (Amersham Biosciences, Arlington Heights, IL) in PBS containing 0.02% Tween 20. The purified material was divided into two batches to produce  
5 the tri-antennary glycan with terminal 2,6-SA moieties and the tri-antennary glycan with terminal 2,6-SA moieties capped with 2,6-SA moieties.

**ST3Gal3 Reaction.** The ST3Gal3 reaction was incubated at 32°C for 24 hours. The reaction contained 1 mg/mL galactosylated EPO, 100 mM Tris-Cl pH 7.2, 150 mM NaCl, 0.02% NaN<sub>3</sub>, 50 mU/mg ST3Gal3, and 3 mM CMP-SA. Figure 151 depicts the HPLC  
10 analysis of glycans released from EPO after this step. Based on glycan and MALDI analysis, approximately 80% of the released glycans were tri-antennary branched structures with terminal 2,3-SA moieties. The remaining 20% of the released glycans were bi-antennary structures with terminal 2,3-SA moieties.

**ST6Gal1 sialylation Reaction following the ST3Gal3 Reaction.** The ST6Gal1  
15 reaction was incubated at 32°C for 24 hours. The reaction contained 1 mg/mL sialylated galactosylated EPO, 100 mM Tris-Cl pH 7.2, 150 mM NaCl, 0.02% NaN<sub>3</sub>, 50 mU/mg ST6Gal1, and 3 mM CMP-SA. Figure 152 depicts the results of HPLC analysis of the glycans released from EPO after the ST6Gal1 reaction. Based on glycan and MALDI analysis, approximately 80% of the tri-antennary branched-glycans contained terminal 2,3-SA  
20 moieties. The remaining 20% of the glycans were bi-antennary with terminal 2,3-SA moieties.

**Superdex 75 Purification.** EPO was purified from the contaminants of the ST3Gal3 reactions by a 1.6 cm x 60 cm Superdex-75 gel filtration chromatography (Amersham Biosciences, Arlington Heights, IL) in PBS containing 0.02% Tween-20.

**Bioassay of Tri-antennary and Bi-antennary Sialylated or PEGylated EPO.** The  
25 activity of the tri-antennary and bi-antennary sialylated EPO glycoforms, and the PEG 10 kDa and 1 kDa bi-antennary glycoforms were assayed using the TF-1 cell line and the MTT viability test, as described above. Figure 153 depicts the results of the MTT cell proliferation assay. At 2 µg/ml EDP, the bi-antennary sialylated EPO had nearly the activity of the control  
30 Epogen, while the tri-antennary sialylated EPO had significantly less activity.

Factor IX19. GlycoPEGylation of Factor IX produced in CHO cells

This example sets forth the preparation of asialoFactor IX and its sialylation with CMP-sialic acid-PEG.

5       **Desialylation of rFactor IX.** A recombinant form of Coagulation Factor IX (rFactor IX) was made in CHO cells. 6000 IU of rFactor IX were dissolved in a total of 12 mL USP H<sub>2</sub>O. This solution was transferred to a Centricon Plus 20, PL-10 centrifugal filter with another 6 mL USP H<sub>2</sub>O. The solution was concentrated to 2 mL and then diluted with 15 mL 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub> and then reconcentrated.

10      The dilution/concentration was repeated 4 times to effectively change the buffer to a final volume of 3.0 mL. Of this solution, 2.9 mL (about 29 mg of rFactor IX) was transferred to a small plastic tube and to it was added 530 mU  $\alpha$ 2-3,6,8-Neuraminidase—agarose conjugate (*Vibrio cholerae*, Calbiochem, 450  $\mu$ L). The reaction mixture was rotated gently for 26.5 hours at 32 °C. The mixture was centrifuged 2 minutes at 10,000 rpm and the supernatant

15      was collected. The agarose beads (containing neuraminidase) were washed 6 times with 0.5 mL 50 mM Tris-HCl pH 7.12, 1 M NaCl, 0.05% NaN<sub>3</sub>. The pooled washings and supernatants were centrifuged again for 2 minutes at 10,000 rpm to remove any residual agarose resin. The pooled, desialylated protein solution was diluted to 19 mL with the same buffer and concentrated down to ~ 2 mL in a Centricon Plus 20 PL-10 centrifugal filter. The

20      solution was twice diluted with 15 mL of 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% NaN<sub>3</sub> and reconcentrated to 2 mL. The final desialylated rFactor IX solution was diluted to 3 mL final volume (~10 mg/mL) with the Tris Buffer. Native and desialylated rFactor IX samples were analyzed by IEF-Electrophoresis. Isoelectric Focusing Gels (pH 3-7) were run using 1.5  $\mu$ L (15  $\mu$ g) samples first diluted with 10  $\mu$ L Tris buffer and mixed with 12  $\mu$ L

25      sample loading buffer. Gels were loaded, run and fixed using standard procedures. Gels were stained with Colloidal Blue Stain (Figure 154), showing a band for desialylated Factor IX.

30       **Preparation of PEG (1 kDa and 10 kDa)-SA-Factor IX.** Desialylated rFactor-IX (29 mg, 3 mL) was divided into two 1.5 mL (14.5 mg) samples in two 15 mL centrifuge tubes. Each solution was diluted with 12.67 mL 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% NaN<sub>3</sub> and either CMP-SA-PEG-1k or 10k (7.25  $\mu$ mol) was added. The tubes were

inverted gently to mix and 2.9 U ST3Gal3 (326  $\mu$ L) was added (total volume 14.5 mL). The tubes were inverted again and rotated gently for 65 hours at 32 °C. The reactions were stopped by freezing at -20 °C. 10  $\mu$ g samples of the reactions were analyzed by SDS-PAGE. The PEGylated proteins were purified on a Toso Haas Biosep G3000SW (21.5 x 30 cm, 13  $\mu$ m) HPLC column with Dulbecco's Phosphate Buffered Saline, pH 7.1 (Gibco), 6 mL/min. The reaction and purification were monitored using SDS Page and IEF gels. Novex Tris-Glycine 4-20% 1 mm gels were loaded with 10  $\mu$ L (10  $\mu$ g) of samples after dilution with 2  $\mu$ L of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN<sub>3</sub> buffer and mixing with 12  $\mu$ L sample loading buffer and 1  $\mu$ L 0.5 M DTT and heated for 6 minutes at 85 °C. Gels were stained with Colloidal Blue Stain (Figure 155) showing a band for PEG (1 kDa and 10 kDa)-SA-Factor IX.

#### 20. Direct Sialyl-GlycoPEGylation of Factor IX

This example sets forth the preparation of sialyl-PEGylation of Factor IX without prior sialidase treatment.

**Sialyl-PEGylation of Factor-IX with CMP-SA-PEG-(10 kDa).** Factor IX (1100 IU), which was expressed in CHO cells and was fully sialylated, was dissolved in 5 mL of 20 mM histidine, 520 mM glycine, 2% sucrose, 0.05% NaN<sub>3</sub> and 0.01% polysorbate 80, pH 5.0. The CMP-SA-PEG-(10 kDa) (27 mg, 2.5  $\mu$ mol) was then dissolved in the solution and 1 U of ST3Gal3 was added. The reaction was complete after gently mixing for 28 hours at 32°C. The reaction was analyzed by SDS-PAGE as described by Invitrogen. The product protein was purified on an Amersham Superdex 200 (10 x 300 mm, 13  $\mu$ m) HPLC column with phosphate buffered saline, pH 7.0 (PBS), 1 mL/min. R<sub>t</sub> = 9.5 min.

**Sialyl-PEGylation of Factor-IX with CMP-SA-PEG-(20 kDa).** Factor IX (1100 IU), which was expressed in CHO cells and was fully sialylated, was dissolved in 5 mL of 20 mM histidine, 520 mM glycine, 2% sucrose, 0.05% NaN<sub>3</sub> and 0.01% polysorbate 80, pH 5.0. The CMP-SA-PEG-(20 kDa) (50 mg, 2.3  $\mu$ mol) was then dissolved in the solution and CST-II was added. The reaction mixture was complete after gently mixing for 42 hours at 32°C. The reaction was analyzed by SDS-PAGE as described by Invitrogen.



The product protein was purified on an Amersham Superdex 200 (10 x 300 mm, 13  $\mu$ m) HPLC column with phosphate buffered saline, pH 7.0 (Fisher), 1 mL/min.  $R_t$  = 8.6 min.

## 21. Sialic Acid Capping of GlycoPEGylated Factor IX

5 This examples sets forth the procedure for sialic acid capping of sialyl-glycoPEGylated peptides. Here, Factor-IX is the exemplary peptide.

**Sialic acid capping of N-linked and O-linked Glycans of Factor-IX-SA-PEG (10 kDa).** Purified r-Factor-IX-PEG (10 kDa) (2.4 mg) was concentrated in a Centricon<sup>®</sup> Plus 20 PL-10 (Millipore Corp., Bedford, MA) centrifugal filter and the buffer was changed to 50  
10 mM Tris-HCl pH 7.2, 0.15 M NaCl, 0.05% NaN<sub>3</sub> to a final volume of 1.85 mL. The protein solution was diluted with 372  $\mu$ L of the same Tris buffer and 7.4 mg CMP-SA (12  $\mu$ mol) was added as a solid. The solution was inverted gently to mix and 0.1 U ST3Gal1 and 0.1 U ST3Gal3 were added. The reaction mixture was rotated gently for 42 hours at 32 °C.

A 10  $\mu$ g sample of the reaction was analyzed by SDS-PAGE. Novex Tris-Glycine 4-  
15 12% 1 mm gels were performed and stained using Colloidal Blue as described by Invitrogen. Briefly, samples, 10  $\mu$ L (10  $\mu$ g), were mixed with 12  $\mu$ L sample loading buffer and 1  $\mu$ L 0.5 M DTT and heated for 6 minutes at 85 °C (Figure 156, lane 4).

## Factor VIIa

## 20 22. GlycoPEGylation of Recombinant Factor VIIa produced in BHK cells

This example sets forth the PEGylation of recombinant Factor VIIa made in BHK cells.

**Preparation of Asialo-Factor VIIa.** Recombinant Factor VIIa was produced in BHK cells (baby hamster kidney cells). Factor VIIa (14.2 mg) was dissolved at 1 mg/ml in  
25 buffer solution (pH 7.4, 0.05 M Tris, 0.15 M NaCl, 0.001 M CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub>) and was incubated with 300 mU/mL sialidase (*Vibrio cholera*)-agarose conjugate for 3 days at 32 °C. To monitor the reaction a small aliquot of the reaction was diluted with the appropriate buffer and an IEF gel performed according to Invitrogen procedures (Figure 157). The mixture was centrifuged at 3,500 rpm and the supernatant was collected. The resin was washed three  
30 times (3x2 mL) with the above buffer solution ( pH 7.4, 0.05 M Tris, 0.15 M NaCl, 0.05% NaN<sub>3</sub>) and the combined washes were concentrated in a Centricon-Plus-20. The remaining

solution was buffer exchanged with 0.05 M Tris (pH 7.4), 0.15 M NaCl, 0.05% NaN<sub>3</sub> to a final volume of 14.4 mL.

**Preparation of Factor VIIa-SA-PEG (1 kDa and 10 kDa).** The desialylation of Factor VIIa solution was split into two equal 7.2 ml samples. To each sample was added either CMP-SA-5-PEG(1 kDa) (7.4 mg) or CMP-SA-5-PEG(10 kDa) (7.4 mg). ST3Gal3 (1.58U) was added to both tubes and the reaction mixtures were incubated at 32°C for 96 hrs. The reaction was monitored by SDS-PAGE gel using reagents and conditions described by Invitrogen. When the reaction was complete, the reaction mixture was purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The combined fractions containing the product were concentrated at 4°C in Centricon-Plus-20 centrifugal filters (Millipore, Bedford, MA) and the concentrated solution reformulated to yield 1.97 mg (bicinchoninic acid protein assay, BCA assay, Sigma-Aldrich, St. Louis MO) of Factor VIIa-PEG. The product of the reaction was analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples were dialyzed against water and analyzed by MALDI-TOF. Figure 158 shows the MALDI results for native Factor VIIa. Figure 159 contains the MALDI results for Factor VIIa PEGylated with 1 kDa PEG where peak of Factor VIIa PEGylated with 1 kDa PEG is evident. Figure 160 contains the MALDI results for Factor VIIa PEGylated with 10 kDa PEG where a peak for Factor VIIa PEGylated with 10 kDa PEG is evident. Figure 161 depicts the SDS-PAGE analysis of all of the reaction products, where a band for Factor VIIa-SA-PEG (10 kDa) is evident.

### Follicle Stimulating Hormone (FSH)

#### 23. GlycoPEGylation of human pituitary-derived FSH

This example illustrates the assembly of a conjugate of the invention. Follicle Stimulating Hormone (FSH) is desialylated and then conjugated with CMP-(sialic acid)-PEG.

**Desialylation of Follicle Stimulating Hormone.** Follicle Stimulating Hormone (FSH) (Human Pituitary, Calbiochem Cat No. 869001), 1 mg, was dissolved in 500 µL 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>. This solution, 375 µL, was transferred to a small plastic tube and to it was added 263 mU Neuraminidase II (*Vibrio cholerae*). The reaction mixture was shaken gently for 15 hours at 32 °C. The reaction mixture was added to

N-(*p*-aminophenyl)oxamic acid-agarose conjugate, 600  $\mu$ L, pre-equilibrated with 50 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.05% NaN<sub>3</sub> and gently rotated 6.5 hours at 4 °C. The suspension was centrifuged for 2 minutes at 14,000 rpm and the supernatant was collected. The beads were washed 5 times with 0.5 mL of the buffer and all supernatants were pooled.

5 The enzyme solution was dialyzed (7000 MWCO) for 15 hours at 4 °C with 2 L of a solution containing 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>, and then twice for 4 hours at 4 °C into 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The solution was concentrated to 2  $\mu$ g/ $\mu$ L by Speed Vac and stored at -20 °C. Reaction samples were analyzed by IEF gels (pH 3-7) (Invitrogen) (Figure 162).

10 **Preparation of human pituitary-derived SA-FSH and PEG-SA-Follicle Stimulating Hormone.** Desialylated FSH (100  $\mu$ g, 50  $\mu$ L) and CMP-sialic acid or CMP-SA-PEG (1 kDa or 10 kDa) (0.05  $\mu$ mol) were dissolved in 13.5  $\mu$ L H<sub>2</sub>O (adjusted to pH 8 with NaOH) in 0.5 mL plastic tubes. The tubes were vortexed briefly and 40 mU ST3Gal3 (36.5  $\mu$ L) was added (total volume 100  $\mu$ L). The tubes were vortexed again and shaken gently for

15 24 hours at 32 °C. The reactions were stopped by freezing at -80 °C. Reaction samples of 15  $\mu$ g were analyzed by SDS-PAGE (Figure 163), IEF gels (Figure 164) and MALDI-TOF. Native FSH was also analyzed by SDS-PAGE (Figure 165)

**Analysis of SDS PAGE and IEF Gels of Reaction Products.** Novex Tris-Glycine 8-16% 1 mm gels for SDS PAGE analysis were purchased from Invitrogen. 7.5  $\mu$ L (15  $\mu$ g)

20 of FSH reaction samples were diluted with 5  $\mu$ L of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN<sub>3</sub> buffer, mixed with 15  $\mu$ L sample loading buffer and 1  $\mu$ L 9 M  $\mu$ -mercaptoethanol and heated for 6 minutes at 85 °C. Gels were run as directed by Invitrogen and stained with Colloidal Blue Stain (Invitrogen).

FSH samples (15  $\mu$ g) were diluted with 5  $\mu$ L Tris buffer and mixed with 15  $\mu$ L

25 sample loading buffer (Figure 162). The samples were then applied to Isoelectric Focusing Gels (pH 3-7) (Invitrogen) (Figure 165). Gels were run and fixed as directed by Invitrogen and then stained with Colloidal Blue Stain.

24. GlycoPEGylation of recombinant FSH produced recombinantly in CHO cells

This example illustrates the assembly of a conjugate of the invention. Desialylated FSH was conjugated with CMP-(sialic acid)-PEG.

5       **Preparation of recombinant Asialo-Follicle Stimulation Hormone.** Recombinant Follicle Stimulation Hormone (rFSH) produced from CHO was used in these studies. The 7,500 IU of rFSH was dissolved in 8 mL of water. The FSH solution was dialyzed in 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub> and concentrated to 500 µL in a Centricon Plus 20 centrifugal filter. A portion of this solution (400 µL) (~ 0.8 mg FSH) was transferred to a  
10   small plastic tube and to it was added 275 mU Neuraminidase II (*Vibrio cholerae*). The reaction mixture was mixed for 16 hours at 32 °C. The reaction mixture was added to prewashed N-(*p*-aminophenyl)oxamic acid-agarose conjugate (800 µL) and gently rotated for 24 hours at 4 °C. The mixture was centrifuged at 10,000 rpm and the supernatant was collected. The beads were washed 3 times with 0.6 mL Tris-EDTA buffer, once with 0.4 mL  
15   Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants were pooled. The supernatant was dialyzed at 4 °C against 2 L of 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution was then concentrated to 420 µL in a Centricon Plus 20 centrifugal filter and stored at -20 °C.

20       Native and desialylated rFSH samples were analyzed by SDS-PAGE and IEF (Figure 166). Novex Tris-Glycine 8-16% 1 mm gels were purchased from Invitrogen. Samples (7.5 µL, 15 µg) samples were diluted with 5 µL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN<sub>3</sub> buffer, mixed with 15 µL sample loading buffer and 1 µL 9 M β-mercaptoethanol and heated for 6 minutes at 85 °C. Gels were run as directed by Invitrogen  
25   and stained with Colloidal Blue Stain (Invitrogen). Isoelectric Focusing Gels (pH 3-7) were purchased from Invitrogen. Samples (7.5 µL, 15 µg) were diluted with 5 µL Tris buffer and mixed with 15 µL sample loading buffer. Gels were loaded, run and fixed as directed by Invitrogen. Gels were stained with Colloidal Blue Stain. Samples of native and desialylated FSH were also dialyzed against water and analyzed by MALDI-TOF.

30       **Sialyl-PEGylation of recombinant Follicle Stimulation Hormone.** Desialylated FSH (100 µg, 54 µL) and CMP-SA-PEG (1 kDa or 10 kDa) (0.05 µmol) were dissolved in 28

μL 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2 in 0.5 mL plastic tubes. The tubes were vortexed briefly and 20 mU of ST3Gal3 was added (total volume 100 μL). The tubes were vortexed again, mixed gently for 24 hours at 32 °C and the reactions stopped by freezing at -80 °C. Samples of this reaction were analyzed as described above by SDS-PAGE gels

5 (Figure 167), IEF gels (Figure 168) and MALDI-TOF MS.

MALDI was also performed on the PEGylated rFSH. During ionization, SA-PEG is eliminated from the N-glycan structure of the glycoprotein. Native FSH gave a peak at 13928; AS-rFSH (13282); resialylated r-FSH (13332); PEG1000-rFSH (13515; 14960 (1); 16455 (2); 17796 (3); 19321 (4)); and PEG 10000 (23560 (1); 34790 (2); 45670 (3); and  
10 56760 (4)).

#### 25. Pharmacokinetic Study of GlycoPEGylated FSH

This example sets forth the *in vivo* testing of the pharmacokinetic properties glycoPEGylated Follicle Stimulating Hormone (FSH) prepared according to the methods of  
15 the invention as compared to non-PEGylated FSH.

FSH, FSH-SA-PEG (1 kDa) and FSH-SA-PEG (10 kDa) were radioiodinated using standard conditions (Amersham Biosciences, Arlington Heights, IL) and formulated in phosphate buffered saline containing 0.1% BSA. After dilution in phosphate buffer to the appropriate concentration, each of the test FSH proteins (0.4 μg, each) was injected  
20 intravenously into female Sprague Dawley rats (250-300 g body weight) and blood drawn at time points from 0 to 80 hours. Radioactivity in blood samples was analyzed using a gamma counter and the pharmacokinetics analyzed using standard methods (Figure 169). FSH was cleared from the blood much more quickly than FSH-PEG(1 kDa), which in turn was clear somewhat more quickly than FSH-PEG(10 kDa).

25

#### 26. Sertoli Cell Bioassay for *In Vitro* Activity of GlycoPEGylated FSH

This example sets forth a bioassay for follicle stimulating hormone (FSH) activity based on cultured Sertoli cells. This assay is useful to determine the bioactivity of FSH after glycan remodeling, including glycoconjugation.

30 This bioassay is based on the dose-response relationship that exists between the amount of estradiol produced when FSH, but not lutenizing hormone (LH), is added to

cultured Sertoli cells obtained from immature old rats. Exogenous testosterone is converted to 17 $\beta$ -estradiol in the presence of FSH.

Seven to 10 days old Sprague-Dawley rats were used to obtain Sertoli cells. After sacrifice, testes were decapsulated and tissue was dispersed by incubation in collagenase (1 mg/ml), trypsin (1mg/ml), hyaluronidase (1 mg/ml) and DNases (5  $\mu$ g/ml) for 5 to 10 min. The tubule fragments settled to the bottom of the flask and were washed in PBS (1x). The tubule fragments were reincubated for 20 min with a media containing the same enzymes: collagenase (1 mg/ml), trypsin (1mg/ml), hyaluronidase (1 mg/ml) and DNases (5  $\mu$ g/ml).

The tubule fragments were homogenized and plated into a 24 well plate in a serum free media.  $5 \times 10^5$  cells were dispersed per well. After 48h incubation at 37° C and 5% CO<sub>2</sub>, fresh media was added to the cells. Composition of the serum free media: DMEM (1 vol), Ham's F10 nutrient mixture (1 vol), insulin 1  $\mu$ g/ml, Transferrin 5  $\mu$ g/ml, EGF 10 ng/ml, T4 20 pg/ml, Hydrocortisone 10<sup>-8</sup> M, Retinoic acid 10<sup>-6</sup> M.

The stimulation experiment consists of a 24 hour incubation with standard FSH or samples at 37°C and 5% CO<sub>2</sub>. The mean intra-assay coefficient of variation is 9% and the mean inter-assay coefficient of variation is 11%.

The 17B-estradiol Elisa Kit DE2000 (R&D Systems, Minneapolis, MN) was used to quantify the level of estradiol after incubation with FSH, FSH-SA-PEG (1 kDa) and FSH-SA-PEG (10 kDa).

The procedure was as follows: 100  $\mu$ l of Estradiol Standard (provided with kit and prepared as per instructions with kit) or sample was pipetted into wells of 17B-estradiol Elisa plate(s); 50  $\mu$ l of 17B-estradiol Conjugate (provided with kit, prepared as per instructions with kit) was added to each well; 50  $\mu$ l of 17B-estradiol antibody solution (provided with kit and prepared as per instructions with kit) was added to each well; plates were incubated for 2 hour at room temperature at 200 rpm; the liquid was aspirated from each well; the wells were washed 4 times using the washing solution; all the liquid was removed from the wells; 200  $\mu$ l of pNPP Substrate (provided with kit and prepared as per instructions with kit) was added to all wells and incubated for 45 min; 50  $\mu$ l of Stop solution (provided with kit and prepared as per instructions with kit) was added and the plates were read it at 405 nm (Figure 170). While FSH-PEG(10 kDa) exhibited a modest stimulation of Sertoli cells, at 1  $\mu$ g/ml, FSH-PEG(1 kDa) stimulated Sertoli cells up to 50% more than unPEGylated FSH.

### 27. Steelman-Pohley Bioassay of *In Vivo* Activity of GlycoPEGylated FSH

In this example, the Steelman-Pohley bioassay (Steelman and Pohley, 1953, Endocrinology 53:604-615) was used to determine the *in vivo* activity of glycoPEGylated FSH. The Steelman-Pohley assay uses the change in ovary weight of a rat to measure the *in vivo* activity of FSH that is coinjected with human chorionic gonadotropin.

The Steelman-Pohley bioassay was performed according to the protocol described in Christin-Maitre et al. (2000, Methods 21:51-57). Seventy female Sprague-Dawley Rats (Charles River Laboratories, Wilmington, MA), aged 21 to 22 days, were housed in the testing facility for at least 5 days before the beginning the assay procedure. Throughout the procedure, the animal room was climate controlled at 18 to 26°C, 30 to 70% relative humidity, and 12 hr. artificial light/12 hr. dark. All animals were fed Certified Rodent Chow (Harlan Teklad, Madison WI) or the equivalent, and water, both *ad libitum*. Animal procedures were performed at Calvert Preclinical Services, Inc. (Olyphant, PA).

Recombinant FSH was expressed in CHO cells, purified by standard techniques and glycoPEGylated with PEG (1 kDa). The rats were divided into seven test groups, with ten animals per group. On days -1 and 0, animals of all groups were subcutaneously injected with 20 I.U. of human chorionic gonadotropin (HCG) in 0.5 ml of 0.9 % NaCl. On days 1, 2 and 3, the control animals were subcutaneously injected with a dose of 0.5 ml containing 20 I.U. HCG in 0.9% NaCl, while in the other groups, the HCG dose was augmented with either rFSH or rFSH-SA-PEG (1 kDa) at either 0.14 µg, 0.4 µg or 1.2 µg per dose. On day 4, the animals were euthanized by CO<sub>2</sub> inhalation. The ovaries were removed, trimmed and weighted. The average ovary weight was determined for each group.

Figure 171 presents the average ovary weight of the test groups on day 4. The groups receiving HCG alone (control) or the low dose (0.14 µg) of either rFSH or rFSH-SA-PEG (1 kDa) had ovary weights that were roughly equivalent. The groups receiving the medium (0.4 µg) or high (1.2 µg) doses of rFSH or rFSH-SA-PEG (1 kDa) had ovary weights roughly twice that of the control group. At the medium dose (0.4 µg), the glycoPEGylated rFSH had roughly the same *in vivo* activity (as determined by ovary weight) as the unPEGylated rFSH.

At the high dose (1.2 µg), the glycoPEGylated rFSH had somewhat higher *in vivo* activity than the unPEGylated rFSH.

### G-CSF

#### 5                    28. GlycoPEGylation of G-CSF produced in CHO cells

**Preparation of Asialo-Granulocyte-Colony Stimulation Factor (G-CSF).** G-CSF produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub> and concentrated to 500 µL in a Centricon Plus 20 centrifugal filter. The solution is incubated with 300 mU/mL Neuraminidase II (*Vibrio cholerae*) for 16  
10 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed. The reaction mixture is then added to prewashed N-(*p*-aminophenyl)oxamic acid-agarose conjugate (800 µL/mL reaction volume) and the washed beads gently rotated for 24 hours at 4 °C. The mixture is centrifuged at 10,000 rpm and the supernatant was collected. The beads are washed 3 times with Tris-EDTA buffer,  
15 once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants are pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 centrifugal filter and stored at -20 °C. The conditions for the IEF gel were run according to  
20 the procedures and reagents provided by Invitrogen. Samples of native and desialylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of G-CSF-(alpha2,3)-Sialyl-PEG.** Desialylated G-CSF was dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST3Gal1 at 32°C for 2 days.  
25 To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas  
30 G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis



according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of G-CSF-(alpha2,8)-Sialyl-PEG.** G-CSF produced in CHO cells, which contains an alpha2,3-sialylated O-linked glycan, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of CST-II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction has CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of G-CSF-(alpha2,6)-Sialyl-PEG.** G-CSF, containing only O-linked GalNAc, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST6GalNAcI or II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction has CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

G-CSF produced in CHO cells was treated with Arthrobacter sialidase and was then purified by size exclusion on Superdex75 and was treated with ST3Gal1 or ST3 Gal2 and then with CMP-SA-PEG 20Kda. The resulting molecule was purified by ion-exchange and

gel filtration and analysis by SDS PAGE demonstrated that the PEGylation was complete. This is the first demonstration of glycoPEGylation of an O-linked glycan.

#### Glucocerebrosidase

##### 5                   29. Glucocerebrosidase-mannose-6-phosphate produced in CHO cells

This example sets forth the procedure to glycoconjugate mannose-6-phosphate to a peptide produced in CHO cells such as glucocerebrosidase.

**Preparation of asialo-glucoceramide.** Glucocerebrosidase produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is  
10 incubated with 300 mU/mL sialidase-agarose conjugate for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel and SDS-PAGE performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The beads are washed 3 times with Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer, and once with 0.2 mL of the Tris-EDTA buffer.  
15 All supernatants are pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 centrifugal filter. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed  
20 against water and analyzed by MALDI-TOF MS.

**Preparation of Glucocerebrosidase-SA-linker-Mannose-6-phosphate (procedure 1).** Asialo-glucocerebrosidase from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-linker-Man-6-phosphate and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the  
25 incorporation of sialic acid-linker-Man-6-phosphate, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas TSK-Gel-3000 analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. When the reaction is complete, the reaction mixture is purified  
30 using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using

SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Glucocerebrosidase-SA-linker-Mannose-6-phosphate (procedure 2).** Glucocerebrosidase, produced in CHO but incompletely sialylated, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-linker-Man-6-phosphate and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid-linker-Man-6-phosphate, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas TSK-Gel-3000 analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

### 30. Glucocerebrosidase-transferrin

This example sets forth the procedures for the glycoconjugation of proteins, and in particular, transferrin is glycoconjugated to glucocerebrosidase. The GlcNAc-ASN structures are created on glucoceraminidase, and Transferrin-SA-Linker-Gal-UDP is conjugated to GNDF GlcNAc-ASN structures using galactosyltransferase.

**Preparation of GlcNAc-glucocerebrosidase (Cerezyme™).** Cerezyme™ (glucocerebrosidase) produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is incubated with 300 mU/mL Endo-H-agarose conjugate for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel and SDS-PAGE performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The beads are washed 3 times with Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants are pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice

more against 50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 centrifugal filter. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Transferrin-SA-Linker-Gal-glucocerebrosidase.** Transferrin-SA-Linker-Gal-UDP from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL GlcNAc-glucocerebrosidase and 0.1 U/mL of galactosyltransferase at 32°C for 2 days. To monitor the incorporation of glucocerebrosidase, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1) and the product detected by UV absorption. The reaction mixture is then purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

### GM-CSF

#### 31. Generation and PEGylation of GlcNAc-ASN Structures: GM-CSF produced in *Saccharomyces*

This example sets forth the preparation of Tissue-type Activator with PEGylated GlcNAc-Asn structures.

Recombinant GM-CSF expressed in yeast is expected to contain 2 N-linked and 2 O-linked glycans. The N-linked glycans should be of the branched mannan type. This recombinant glycoprotein is treated with an endoglycosidase from the group consisting of endoglycosidase H, endoglycosidase-F1, endoglycosidase-F2, endoglycosidase-F3, endoglycosidase-M either alone or in combination with mannosidases I, II and III to generate GlcNAc nubs on the asparagine (Asn) residues on the peptide/protein backbone.

The GlcNAc-Asn structures on the peptide/protein backbone is then be modified with galactose or galactose-PEG using UDP-galactose or UDP-galactose-6-PEG, respectively, and a galactosyltransferase such as GalT1. In one case the galactose-PEG is the terminal residue.

In the second case the galactose is further modified with SA-PEG using a CMP-SA-PEG donor and a sialyltransferase such as ST3GalIII. In another embodiment the GlcNAc-Asn structures on the peptide/protein backbone can be galactosylated and sialylated as described above, and then further sialylated using CMP-SA-PEG and an  $\alpha$ 2,8-sialyltransferase such as the enzyme encoded by the *Campylobacter jejuni* cst-II gene.

### Herceptin™

#### 32. Glycoconjugation of mithramycin to Herceptin™

This example sets forth the procedures to glycoconjugate a small molecule, such as mithramycin to Fc region glycans of an antibody molecule produced in mammalian cells. Here, the antibody Herceptin™ is used, but one of skill in the art will appreciate that the method can be used with many other antibodies.

**Preparation of Herceptin™-Gal-linker-mithramycin.** Herceptin™ is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM UDP-galactose-linker-mithramycin and 0.1 U/mL of galactosyltransferase at 32°C for 2 days to introduce the mithramycin in the Fc region glycans. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

Interferon  $\alpha$  and Interferon  $\beta$ 33. GlycoPEGylation of Proteins expressed in Mammalian or Insect Systems:  
EPO, Interferon  $\alpha$  and Interferon  $\beta$ 

5 This example sets forth the preparation of PEGylated peptides that are expressed in mammalian and insect systems.

**Preparation of acceptor from mammalian expression systems.** The peptides to be glycoPEGylated using CMP-sialic acid PEG need to have glycans terminating in galactose. Most peptides from mammalian expression systems will have terminal sialic acid that first  
10 needs to be removed.

**Sialidase digestion.** The peptide is desialylated using a sialidase. A typical procedure involves incubating a 1 mg/mL solution of the peptide in Tris-buffered saline, pH 7.2, with 5 mM CaCl<sub>2</sub> added, with 0.2 U/mL immobilized sialidase from *Vibrio cholera* (Calbiochem) at 32°C for 24 hours. Microbial growth can be halted either by sterile filtration  
15 or the inclusion of 0.02% sodium azide. The resin is then removed by centrifugation or filtration, and then washed to recover entrapped peptide. At this point, EDTA may be added to the solution to inhibit any sialidase that has leached from the resin.

**Preparation from insect expression systems.** EPO, interferon-alpha, and interferon-beta may also be expressed in non-mammalian systems such as yeast, plants, or  
20 insect cells. The peptides to be glycoPEGylated using CMP-sialic acid PEG need to have glycans terminating in galactose. The majority of the N-glycans on peptides expressed in insect cells, for example, are the trimannosyl core. These glycans are first built out to glycans terminating in galactose before they are acceptors for sialyltransferase.

**Building acceptor glycans from trimannosyl core.** Peptide (1 mg/mL) in Tris-  
25 buffered saline, pH 7.2, containing 5 mM MnCl<sub>2</sub>, 5 mM UDP-glcNAc, 0.05 U/mL GLCNACT I, 0.05 U/mL GLCNACT II, is incubated at 32°C for 24 hours or until the reaction is substantially complete. Microbial growth can be halted either by sterile filtration or the inclusion of 0.02% sodium azide. After buffer exchange to remove UDP and other small molecules, UDP-galactose and MnCl<sub>2</sub> are each added to 5 mM, galactosyltransferase is  
30 added to 0.05 U/mL, and is incubated at 32°C for 24H or until the reaction is substantially

complete. Microbial growth can be halted either by sterile filtration or the inclusion of 0.02% sodium azide. The peptides are then ready for glycoPEGylation.

**Building O-linked glycans.** A similar strategy may be employed for interferon alpha to produce enzymatically the desired O-glycan Gal-GalNAc. If necessary, GalNAc linked to serine or threonine can be added to the peptide using appropriate peptide GalNAc transferases (e.g. GalNAc T1, GalNAc T2, T3, T4, etc. ) and UDP-GalNAc. Also, if needed, galactose can be added using galactosyltransferase and UDP-galactose.

**GlycoPEGylation using sialyltransferase.** The glycopeptides (1 mg/mL) bearing terminal galactose in Tris buffered saline + 0.02% sodium azide are incubated with CMP-SA-PEG (0.75 mM) and 0.4 U/mL sialyltransferase (ST3Gal3 or ST3Gal4 for N-glycans on EPO and interferon beta; ST3Gal4, or ST3Gal1 for O-glycans on interferon alpha) at 32°C for 24 hours. Other transferases that may work include the 2,6 sialyltransferase from *Photobacterium damsella*. The acceptor peptide concentration is most preferably in the range of 0.1 mg/mL up to the solubility limit of the peptide. The concentration of CMP-SA-PEG should be sufficient for there to be excess over the available sites, but not so high as to cause peptide solubility problems due to the PEG, and may range from 50 µM up to 5 mM, and the temperature may range from 2°C up to 40°C. The time required for complete reaction will depend on the temperature, the relative amounts of enzyme to acceptor substrate, the donor substrate concentration, and the pH.

#### 34. GlycoPEGylation of Interferon α produced in CHO cells

**Preparation of Asialo-Interferon α.** Interferon alpha produced from CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub> and concentrated to 500 µL in a Centricon Plus 20 centrifugal filter. The solution is incubated with 300 mU/mL Neuraminidase II (*Vibrio cholerae*) for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed. The reaction mixture is then added to prewashed N-(p-aminophenyl)oxamic acid-agarose conjugate (800 µL/mL reaction volume) and the washed beads gently rotated for 24 hours at 4 °C. The mixture is centrifuged at 10,000 rpm and the supernatant was collected. The beads are washed 3 times with Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all

supernatants were pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 centrifugal filter and stored at -20 °C. The conditions for the IEF gel are run according to the procedures and reagents provided by Invitrogen. Samples of native and desialylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Interferon-alpha-(alpha2,3)-Sialyl-PEG.** Desialylated interferon-alpha is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST3Gal1 at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and desialylated Interferon-alpha are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Interferon-alpha-(alpha2,8)-Sialyl-PEG.** Interferon-alpha produced in CHO, which contains an alpha2,3-sialylated O-linked glycan, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of CST-II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction has CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis



according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated interferon-alpha are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Interferon-alpha-(alpha2,6)-Sialyl-PEG.** Interferon-alpha, containing only O-linked GalNAc, was dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST6GalNAcI or II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated interferon-alpha are dialyzed against water and analyzed by MALDI-TOF MS.

### 35. GlycoPEGylation of Interferon-β-1a with PEG (10 kDa) and PEG (20 kDa)

This example illustrates a procedure PEGylate Interferon-β with either PEG (10 kDa) or PEG (20 kDa).

Briefly, Interferon-β-1a (INF-β) was obtained from Biogen (Avonex™). The IFN-β was first purified by Superdex-75 chromatography. The IFN-β was then desialylated with *Vibrio cholerae* sialidase. The INF-β was then PEGylated with SA-PEG (10 kDa) or SA-PEG (20 kDa) and purified with Superdex-200 chromatography.

**Superdex-75 chromatography purification.** INF-β (150 μg) was applied to a Superdex-75 column (Amersham Biosciences, Arlington Heights, IL) and eluted with PBS with 0.5 M NaCl, 0.02 Tween-20, 20 mM histidine and 10% glycerol. The eluant was monitored for absorbance at 280 nm (Figure 172A and 172B) and fractions were collected. Peaks 4 and 5 were pooled, concentrated in an Amicon Ultra 15 spin filter (Millipore, Billerica, MA), and the buffer was exchanged to TBS with 5 mM CaCl<sub>2</sub>, 0.02% Tween-20, 20 mM histidine and 10% glycerol.

**Sialidase Reaction.** The INF- $\beta$  was then desialyated with *Vibrio cholera* sialidase (70 mU/ml, CALBIOCHEM®, EMD Biosciences, Inc., San Diego, CA) on agarose in TBS with 5 mM CaCl<sub>2</sub>, 0.02% Tween-20, 20 mM histidine and 10% glycerol. The reaction was carried out at 32°C for 18 hours. The INF- $\beta$  was removed from the agarose with a 0.22  $\mu$ m Spin-X™ filter (Corning Technology, Inc., Norcross, GA). Figure 173A depicts the MALDI analysis of glycans released from native INF- $\beta$ . The native INF- $\beta$  has many glycoforms containing terminal sialic acid moieties. Figure 173B depicts the MALDI analysis of glycans released from desialylated INF- $\beta$ . The desialylated INF- $\beta$  has primarily one glycoform which is bi-antennary with terminal galactose moieties.

**Lectin Dot-Blot Analysis of Sialylation.** Samples of the INF- $\beta$  from the desialidase reaction were dot-blotted onto nitrocellulose and then blocked with Tris buffered saline (TBS: 0.05M Tris, 0.15M NaCl, pH 7.5) and DIG kit (glycan differentiation kit available from Roche #1 210 238) blocking buffer. Some of the blots were incubated with *Maackia amurensis* agglutinin (MAA) labeled with digoxigenin (DIG) (Roche Applied Science, Indianapolis, IL) to detect  $\alpha$ 2,3-sialylation of INF- $\beta$ . These blots were washed with TBS then incubated with anti-digoxigenin antibody labeled with alkaline phosphatase, then washed again with TBS and developed with NBT/X-phosphate solution, wherein NBT is 4-nitro blue tetrazolium chloride and X-phosphate is 5-bromo-4-chloro-3-indoyl phosphate. The left side of Figure 174 depicts the results of the MAA blot of INF- $\beta$  after the desialylation reaction. The INF- $\beta$  is partially desialylated, as indicated by the decrease in dot development as compared to native INF- $\beta$  in the desialylated samples.

Other blots were incubated with *Erthrina cristagalli* lectin (ECL) labeled with biotin (Vector Laboratories, Burlingame, CA) to detect exposed galactose residues on INF- $\beta$ . After incubation with 2.5  $\mu$ g/ml ECL, the blots were washed in TBS and incubated with streptavidin labeled with alkaline phosphatase. The blots were then washed again and developed. The right side of Figure 174 depicts the ECL blot after development. The increased intensity of the dot of desialylated INF- $\beta$  as compared to the native INF- $\beta$  indicate more exposed galactose moieties and therefore extensive desialylation.

**PEGylation of Desialylated INF- $\beta$  with SA-PEG (10 kDa).** Desialylated INF- $\beta$  (0.05 mg/ml) was PEGylated with ST3Gal3 (50 mU/ml) and CMP-SA-PEG (10 kDa) (250

μM) in an appropriate buffer of TBS + 5 mM CaCl<sub>2</sub>, 0.02% Tween 20, 20 mM histidine, 10% glycerol for 50 hours at 32°C. Figure 175 depicts the SDS-PAGE analysis of the reaction products showing PEGylated INF-β at approximately 98 kDa.

**PEGylation of Desialylated INF-β with SA-PEG (20 kDa).** Desialylated INF-β (0.5 mg/ml) was PEGylated with ST3Gal3 (170 mU/ml) and CMP-SA-PEG (20 kDa) in an appropriate buffer of TBS + 5 mM CaCl<sub>2</sub>, 0.02% Tween 20, 20 mM histidine, 10% glycerol for 50 hours at 32°C. Figure 176 depicts the SDS-PAGE analysis the products of the PEGylation reaction. The PEGylated INF-β has many higher molecular weight bands not found in the unmodified INF-β indicating extensive PEGylation.

**Superdex-200 Purification of INF-β PEGylated with PEG (10 kDa).** The products of the PEGylation reaction were separated on a Superdex-200 column (Amersham Biosciences, Arlington Heights, IL) in PBS with 0.5 NaCl, 0.02 Tween-20, 20 mM histidine and 10% glycerol at 1ml/min and 30 cm/hr flow. The eluant was monitored for absorbance at 280 nm (Figure 177) and fractions were collected. Peaks 3 and 4 were pooled and concentrated in an Amicon Ultra 15 spin filter.

**Bioassay of INF-β PEGylated with PEG (10 kDa).**

The test is inhibition of the proliferation of the lung carcinoma cell line, A549. The A549 cell line are lung carcinoma adherent cells growing in RPMI + 10% FBS at 37°C 5% CO<sub>2</sub>. They can be obtained from ATCC # CCL-185. Wash the cells with 10 ml of PBS and remove the PBS. Add 5 ml of trypsin, incubate for 5 minutes at room temperature or 2 minutes at 37°C. When the cells are detached resuspend into 25 ml of media and count the cells. Dilute the cells at a concentration of 10000 cells/ml and add 200 ul / well (96 wells plate). Incubate for 4 hours at 37°C 5% CO<sub>2</sub>. Prepare 1 ml of IFN B at a concentration of 0.1 ug/ml. Filter it under the hood with a 0.2 μm filter. Add 100 ul per well (8 replicates = 1 lane). Incubate for 3 days (do not let the cells go to confluence). Remove 200 ul of media (only 100ul per well left). Add 25 μl of MTT (Sigma) (5 mg/ml filtered 0.22μm). Incubate for 4 hours at 37°C and 5% CO<sub>2</sub>. Aspirate the media gently and add 100 μl of a mixture of isopropanol (100 ml and 6N HCl. Aspirate up and down to homogenize the crystal violet. Read OD 570nm (remove the background at 630 or 690 nm).

Figure 178 depicts the results of the bioassay of the peaks containing INF- $\beta$  PEGylated with PEG (10 kDa) as eluted from the Superdex-200 column.

**Superdex-200 Purification of INF- $\beta$  PEGylated with PEG (20 kDa).** The products of the PEG (20 kDa) PEGylation reaction were separated on a Superdex-200 column (Amersham Biosciences, Arlington Heights, IL) in PBS with 0.5 NaCl, 0.02 Tween-20, 20 mM histidine and 10% glycerol at 1 ml/min flow. The eluant was monitored for absorbance at 280 nm (Figure 179) and fractions were collected. Peak 3 contained most of the INF- $\beta$  PEGylated with PEG (20 kDa).

**Endotoxin test of INF- $\beta$  PEGylated with PEG (20 kDa).**

Limulus Lysate Test was performed, BioWhittaker # 50-647U

**Table 24.** Results of the endotoxin test of INF- $\beta$  PEGylated with PEG (20 kDa).

	Concentration		
INF- $\beta$ with PEG (20 kDa)	10 EU/ml	0.06 mg/ml	0.16 EU/ $\mu$ g
INF- $\beta$ with PEG (20 kDa)	1 EU/ml	0.07 mg/ml	0.014 EU/ $\mu$ g
Native INF- $\beta$	40 EU/ml	0.1 mg/ml	0.4 EU/ $\mu$ g

Remicade™

36. GlycoPEGylation of Remicade™ antibody

This example sets forth the procedure to glycoPEGylate a recombinant antibody molecule by introducing PEG molecules to the Fc region glycans. Here Remicade™, a TNF-R:IgG Fc region fusion protein, is the exemplary peptide.

**Preparation of Remicade™-Gal-PEG (10 kDa).** Remicade™ is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM UDP-galactose-PEG (10 kDa) and 0.1 U/mL of galactosyltransferase at 32°C for 2 days to introduce the PEG in the Fc region glycans. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

### Rituxan™

#### 37. Glycoconjugation of geldanamycin to Rituxan™

This example sets forth the glycoconjugation of a small molecule, such as geldanamycin, to the Fc region glycans of an antibody produced in CHO cells, such as Rituxan™. Here, the antibody Rituxan™ is used, but one of skill in the art will appreciate that the method can be used with many other antibodies.

**Preparation of Rituxan™-Gal-linker-geldanamycin.** Rituxan™ is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM UDP-galactose-linker-geldanamycin and 0.1 U/mL of galactosyltransferase at 32°C for 2 days to introduce the geldanamycin in the Fc region glycans. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

Rnase38. Remodeling high mannose N-glycans to hybrid and complex N-glycans:  
Bovine pancreatic RNase

This example sets forth the preparation of bovine pancreas RNase with hybrid or  
5 complex N-glycans. The high mannose N-linked glycans of the RNase are enzymatically  
digested and elaborated to create hybrid N-linked glycans. Additionally, the high mannose  
N-linked glycans of the RNase are enzymatically digested and elaborated to create complex  
N-linked glycans.

High mannose structures of *N*-linked oligosaccharides in glycopeptides can be  
10 modified to hybrid or complex forms using the combination of  $\alpha$ -mannosidases and  
glycosyltransferases. This example summarizes the results in such efforts using a simple *N*-  
Glycan as a model substrate.

Ribonuclease B (RNaseB) purified from bovine pancreas (Sigma) is a glycopeptide  
consisting of 124 amino acid residues. It has a single potential *N*-glycosylation site modified  
15 with high mannose structures. Due to its simplicity and low molecular weight (13.7 kDa to  
15.5 kDa), ribonuclease B is a good candidate to demonstrate the feasibility of the *N*-Glycan  
remodeling from high mannose structures to hybrid or complex *N*-linked oligosaccharides.  
The MALDI-TOF spectrum of RNaseB (Figure 180A) and HPLC profile for the  
oligosaccharides cleaved from RNaseB by N-Glycanase (Figure 180B) indicated that, other  
20 than a small portion of the non-modified peptide, the majority of *N*-glycosylation sites of the  
peptide are modified with high mannose oligosaccharides consisting of 5 to 9 mannose  
residues.

**Conversion of high mannose N-Glycans to hybrid N-Glycans.** High mannose *N*-  
Glycans were converted to hybrid *N*-Glycans using the combination of  $\alpha$ 1,2-mannosidase,  
25 GlcNAcT-I ( $\beta$ -1,2-*N*-acetyl glucosaminyl transferase), GalT-I ( $\beta$ 1,4-galactosyltransferase) and  
 $\alpha$ 2,3-sialyltransferase /or  $\alpha$ 2,6-sialyltransferase as shown in Figure 181.

As an example, high mannose structures in RNaseB were successfully converted to  
hybrid structures.

Man<sub>5</sub>GlcNAc<sub>2</sub>-R was obtained from Man<sub>5,9</sub>GlcNAc<sub>2</sub>-R catalyzed by a single  $\alpha$ 1,2-  
30 mannosidase cloned from *Trichoderma reesei* (Figure 182). RNase B (1 g, about 67  $\mu$ mol)  
was incubated at 30°C for 45 hr with 15 mU of the recombinant *T. reesei*  $\alpha$ 1,2-mannosidase

in MES buffer (50 mM, pH 6.5) in a total volume of 10 mL. Man<sub>6-9</sub>GlcNAc<sub>2</sub>-protein structures have been successfully converted to Man<sub>5</sub>GlcNAc<sub>2</sub>-protein with high efficiency by the recombinant mannosidase.

Alternately, Man<sub>5</sub>GlcNAc<sub>2</sub>-R was obtained from Man<sub>5-9</sub>GlcNAc<sub>2</sub>-R catalyzed by a single  $\alpha$ 1,2-mannosidase purified from *Aspergillus saitoi* (Figure 183). RNase B (40  $\mu$ g, about 2.7 nmol) was incubated at 37°C for 42.5 hr with 25  $\mu$ U of the commercial *A. saitoi*  $\alpha$ 1,2-mannosidase (Glyko or CalBioChem) in NaOAc buffer (100 mM, pH 5.0) in a total volume of 20  $\mu$ l. Man<sub>6-9</sub>GlcNAc<sub>2</sub>-protein structures were successfully converted to Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by the commercially available mannosidase. However, a new peak corresponding to the GlcNAc-protein appears in the spectrum, indicating the possible contamination of endoglycosidase H in the preparation. Although several mammalian alpha-mannosidases were required to achieve this step, the fungal  $\alpha$ 1,2-mannosidase was very efficient to remove all  $\alpha$ 1,2-linked mannose residues.

GlcNAcT-I then added a GlcNAc residue to the Man<sub>5</sub>GlcNAc<sub>2</sub>-R (Figure 184). The reaction mixture after the *T. reesei*  $\alpha$ 1,2-mannosidase reaction containing RNase B (600  $\mu$ g, about 40 nmol) was incubated with non-purified recombinant GlcNAcT-I (34 mU) in MES buffer (50 mM, pH 6.5) containing MnCl<sub>2</sub> (20 mM) and UDP-GlcNAc (5 mM) in a total volume of 400  $\mu$ l. at 37°C for 42 hr. A GlcNAc residue was quantitatively added to Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by the recombinant GlcNAcT-I.

A Gal residue was then added using GalT 1 (Figure 185). The reaction mixture after the GnT-I reaction containing RNase B (120  $\mu$ g, about 8 nmol) was incubated at 37°C for 20 hr with 3.3 mU of the recombinant GalT-1 in Tris-HCl buffer (100 mM, pH 7.3) containing UDP-Gal (7.5 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 100  $\mu$ l. A Gal residue was added to about 98% of the GlcNAc-Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by the recombinant GalT 1.

The next step was the addition of a sialic acid using an  $\alpha$ 2,3-sialyltransferase or an  $\alpha$ 2,6-sialyltransferase (Figure 186). As an example, ST3Gal III, an  $\alpha$ 2,3-sialyltransferase was used. The reaction mixture after the GalT-1 reaction containing RNase B (13  $\mu$ g, about 0.87 nmol) was incubated at 37°C for 16 hr with 8.9 mU of recombinant ST3Gal III in Tris-HCl buffer (100 mM, pH 7.3) containing CMP-Sialic acid (5 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 20  $\mu$ l. A sialic acid residue was added to about 90% of the Gal-GlcNAc-

Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by recombinant ST3Gal III using CMP-SA as the donor. The yield can be further improved by adjusting the reaction conditions.

For convenience, no purification or dialysis step was required after each reaction described above. More interesting, GalT 1 and ST3Gal III can be combined in a one-pot reaction. Similar yields were obtained as compared with the separate reactions. The reaction mixture after the GlcNAcT-I reaction containing RNase B (60 µg, about 4 nmol) was incubated at 37°C for 20 hr with 1.7 mU of recombinant GalT 1, 9.8 mU of recombinant ST3Gal III in Tris-HCl buffer (100 mM, pH 7.3) containing UDP-Gal (7.5 mM), CMP-sialic acid (5 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 60 µl.

As shown in Figure 187, SA-PEG (10 kDa) was successfully added to the RNaseB. The reaction mixture after the GalT-1 reaction containing RNase B (6.7 µg, about 0.45 nmol) was dialyzed against H<sub>2</sub>O for 1 hour at room temperature and incubated at 37°C for 15.5 hours with 55 mU of the recombinant ST3Gal III in Tris-HCl buffer (50 mM, pH 7.3) containing CMP-SA-PEG (10 kDa) (0.25 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 20 µl. PEG-modified sialic acid residues were successfully added to the Gal-GlcNAc-Man<sub>5</sub>GlcNAc<sub>2</sub>-peptide by the recombinant ST3Gal III. The yield can be further improved by adjusting the reaction conditions.

**Conversion of high mannose N-Glycans to complex N-Glycans.** To achieve this conversion, a GlcNAcβ1,2Man<sub>3</sub>GlcNAc<sub>2</sub>-peptide intermediate is obtained. As shown in Figure 188, there are at least four feasible routes to carry out the reaction from Man<sub>5</sub>GlcNAc<sub>2</sub>-peptide to this intermediate:

**Route I:** The Man<sub>5</sub>GlcNAc<sub>2</sub>-peptide produced by the fungal α1,2 mannosidase is a substrate of GlcNAc transferase I (GlcNAcT-I, enzyme 2) which adds one GlcNAc. The terminal α1,3- and α1,6-linked mannose residues of GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>-peptide is removed by Golgi α-mannosidase II (ManII, enzyme 5). This route is a part of the natural pathway for the processing of N-linked oligosaccharides carried out in higher organisms.

**Route II:** Two mannose residues are first removed by an α-mannosidase (enzyme 6), then a GlcNAc is added by GlcNAcT-I (enzyme 2). Other than its natural acceptor Man<sub>5</sub>GlcNAc<sub>2</sub>-R, GlcNAcT-I can also recognize Man<sub>3</sub>GlcNAc<sub>2</sub>-R as its substrate and add one GlcNAc to the mannose core structure to form GlcNAcMan<sub>3</sub>GlcNAc<sub>2</sub>-peptide.



**Route III:** The  $\alpha$ 1,6-linked mannose is removed by an  $\alpha$ 1,6-mannosidase, followed by the addition of GlcNAc by GlcNAcT-I and removal of the terminal  $\alpha$ 1,3-linked mannose by an  $\alpha$ 1,3-mannosidase. From the experimental data obtained, GlcNAcT-I can recognize this  $\text{Man}_4\text{GlcNAc}_2$ -peptide as acceptor and add one GlcNAc residue to form

5  $\text{GlcNAcMan}_4\text{GlcNAc}_2$ -peptide.

**Route IV:** Similar to Route III,  $\alpha$ 1,3-linked mannose is removed by an  $\alpha$ 1,3-mannosidase, followed by GlcNAcT-I reaction. Then the terminal  $\alpha$ 1,6-linked mannose can be removed by an  $\alpha$ 1,6-mannosidase.

After the function of GlcNAcT-I (responsible for the addition of the GlcNAc  $\beta$ 1,2-linked to the  $\alpha$ 1,3-mannose on the mannose core) and GlcNAcT-II (responsible for the addition of a second GlcNAc  $\beta$ 1,2-linked to the  $\alpha$ 1,6-mannose on the mannose core), the  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ -peptide can be processed by GalT 1 and sialyltransferase to form bi-antennary complex N- Glycans. Other GlcNAc transferases such as GlcNAcT-IV, GlcNAcT-V, and/or GlcNAcT-VI (Figure 188 and Figure 189) can also glycosylate the

10 linked to the  $\alpha$ 1,3-mannose on the mannose core) and GlcNAcT-II (responsible for the addition of a second GlcNAc  $\beta$ 1,2-linked to the  $\alpha$ 1,6-mannose on the mannose core), the  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ -peptide can be processed by GalT 1 and sialyltransferase to form bi-antennary complex N- Glycans. Other GlcNAc transferases such as GlcNAcT-IV, GlcNAcT-V, and/or GlcNAcT-VI (Figure 188 and Figure 189) can also glycosylate the

15  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ -peptide. Additional glycosylation by the GalT 1 and sialyltransferases will form multi-antennary complex N-glycans. The enzyme GlcNAcT-III catalyzes the insertion of a bisecting GlcNAc, thus preventing the actions of ManII and subsequent action of transferases GlcNAcT-II, GlcNAcT-IV and GlcNAcT-V.

## 20 Tissue-Type Plasminogen Activator (TPA)

### 39. Fucosylation of TPA to create Sialyl Lewis X

This example sets forth the preparation of Tissue Tissue-type Plasminogen Activator (TPA) with N-linked sialyl Lewis X antigen.

**Sialylation.** TPA expressed in mammalian cells will often contain a majority of the glycans terminating in sialic acid, but to ensure complete sialylation, it would be beneficial to first perform an *in vitro* sialylation. TPA in a suitable buffer (most preferably between pH 5.5 and 9, for example Tris buffered saline, pH 7.2) is incubated with CMP sialic acid and sialyltransferase for a time sufficient to convert any glycans lacking sialic acid to sialylated species. Typical conditions would be 1 mg/mL TPA, 3 mM CMP sialic acid, 0.02 U/mL

25 glycans terminating in sialic acid, but to ensure complete sialylation, it would be beneficial to first perform an *in vitro* sialylation. TPA in a suitable buffer (most preferably between pH 5.5 and 9, for example Tris buffered saline, pH 7.2) is incubated with CMP sialic acid and sialyltransferase for a time sufficient to convert any glycans lacking sialic acid to sialylated species. Typical conditions would be 1 mg/mL TPA, 3 mM CMP sialic acid, 0.02 U/mL

30 ST3Gal3, 32°C for 24 hours. Microbial growth can be halted either by sterile filtration or the

inclusion of 0.02% sodium azide. The TPA concentration is most preferably in the range 0.1 mg/mL up to the solubility limit of the peptide. The concentration of CMP-SA should be sufficient for there to be excess over the available sites, and might range from 50  $\mu$ M up to 50 mM, and the temperature from 2°C up to 40°C. The time required for complete reaction will depend on the temperature, the relative amounts of enzyme to acceptor substrate, the donor substrate concentration, and the pH. Other sialyltransferases that may be capable of adding sialic acid in 2,3 linkage include ST3Gal4; microbial transferases could also be used.

**Fucosylation.** Typical conditions for fucosylation would be 1 mg/mL TPA, 3 mM GDP-fucose, 0.02 U/mL FTVI, 5 mM MnCl<sub>2</sub>, 32°C for 24H in Tris buffered saline.

Microbial growth can be halted either by sterile filtration or the inclusion of 0.02% sodium azide. The TPA concentration is most preferably in the range 0.1 mg/mL up to the solubility limit of the peptide. The concentration of GDP-fucose should be sufficient for there to be excess over the available sites, and might range from 50  $\mu$ M up to 50 mM, and the temperature from 2°C up to 40°C. The time required for complete reaction will depend on the temperature, the relative amounts of enzyme to acceptor substrate, the donor substrate concentration, and the pH. Other fucosyltransferases that may be capable of making sialyl Lewis x include FTVII, FTV, FTIII, as well as microbial transferases could also be used.

#### 40. Trimming of high mannose to tri-mannose core structure: Tissue-type Plasminogen Activator produced in CHO

This example sets forth the preparation of Tissue-type Plasminogen Activator with a trimannose core by trimming back from a high mannose glycan.

Tissue-type plasminogen activator (TPA) is currently produced in Chinese Hamster Ovary (CHO) cells and contains a low amount of high mannose N-linked oligosaccharide.

The mannoses can be trimmed down using a variety of the specific mannosidases. The first step is to generate Man5GlcNAc2(Fuc0-1) from Man9GlcNAc2(Fuc0-1). This can be done using mannosidase I. Then either GlcNAcT1 (GlcNAc transferase I) is used to make GlcNAc1Man5GlcNAc2(Fuc0-1) or Mannosidase III is used to make Man3GlcNAc2(Fuc0-1). From Man3GlcNAc2(Fuc0-1), GlcNAc1Man3GlcNAc2(Fuc0-1) can be produced using GlcNAcT1 or from GlcNAc1Man5GlcNAc2(Fuc0-1), GlcNAc1Man3GlcNAc2(Fuc0-1) can be produced using Mannosidase II. GlcNAc1Man3GlcNAc2(Fuc0-1) is then converted into

GlcNAc2Man3GlcNAc2(Fuc0-1) using GlcNAcTransferase II (GlcNAcTII). The two terminal GlcNAc residues are then galactosylated using GalTI and then sialylated with SA-PEG using ST3GalIII.

Conversely, TPA can be produce in yeast or fungal systems. Similar processing  
5 would be required for fungal derived material.

#### 41. Generation and PEGylation of GlcNAc-ASN structures: TPA produced in Yeast

This example sets forth the preparation of PEGylated GlcNAc-Asn structures on a  
10 peptide such as TPA expressed in yeast.

Yeast expression is expected to result in a TPA which contains a single N-linked mannan-type structure. This recombinant glycoprotein is first treated with endoglycosidase H to generate GlcNAc structures on the asparagine (Asn) residues on the peptide.

The GlcNAc-Asn structures on the peptide/protein backbone are then be modified  
15 with galactose or galactose-PEG using UDP-galactose or UDP-galactose-6-PEG, respectively, and a galactosyltransferase such as GalT1. In one case, the galactose-PEG is the terminal residue. In the second case, the galactose is further modified with SA-PEG using a CMP-SA-PEG donor and a sialyltransferase such as ST3GalIII. In another embodiment, the GlcNAc-Asn structures on the peptide/protein backbone may be  
20 galactosylated and sialylated as described above, and then further sialylated using CMP-SA-PEG and an  $\alpha$ 2,8-sialyltransferase such as the enzyme encoded by the *Campylobacter jejuni* cst-II gene.

#### Transferrin

#### 25 42. GlycoPEGylation of Transferrin

This example sets forth the preparation of asialotransferrin and its sialylation with PEG-CMP-sialic acid.

**Preparation of Asialo-transferrin.** Human-derived holo-Transferrin, (10 mg) was dissolved in 500  $\mu$ L of 50 mM NaOAc, 5 mM CaCl<sub>2</sub>, pH 5.5. To this solution was added  
30 500 mU Neuraminidase II (*Vibrio cholerae*) and the reaction mixture was shaken gently for 20.5 hours at 37 °C. The reaction mixture was added to the prewashed N-(p-

aminophenyl)oxamic acid-agarose conjugate (600  $\mu$ L) and the washed beads gently rotated for 24 hours at 4 °C. The mixture was centrifuged at 10,000 rpm and the supernatant was collected. The reaction mixture was adjusted to 5 mM EDTA by addition of 100  $\mu$ L of 30 mM EDTA to the washed beads, which were gently rotated for 20 hours at 4 °C. The suspension was centrifuged for 2 minutes at 10,000 rpm and the supernatant was collected. The beads were washed 5 times with 0.35 mL of 50 mM NaOAc, 5 mM  $\text{CaCl}_2$ , 5 mM EDTA, pH 5.5 and all supernatants were pooled. The enzyme solution was dialyzed twice at 4 °C into 15 mM Tris-HCl, 1 M NaCl, pH 7.4. 0.3 mL of the transferrin solution (3.3 mL total) was removed and dialyzed twice against water. The remainder was dialyzed twice more at 4 °C against phosphate buffered saline. The dialyzed solution was stored at -20 °C. Protein samples were analyzed by IEF Electrophoresis. Samples (9  $\mu$ L, 25  $\mu$ g) were diluted with 16  $\mu$ L Tris buffer and mixed with 25  $\mu$ L of the sample loading buffer and applied to Isoelectric Focusing Gels (pH 3-7). Gels were run and fixed using standard procedures. Gels were stained with Colloidal Blue Stain.

**Sialyl-PEGylation of asialo-Transferrin.** Desialylated transferrin (250  $\mu$ g) and CMP-sialic acid or CMP-SA-PEG (1 kDa or 10 kDa)(0.05  $\mu$ mol) were dissolved in 69  $\mu$ L 50 mM Tris-HCl, 0.15 M NaCl, 0.05%  $\text{NaN}_3$ , pH 7.2 in 1.5 mL plastic tubes. The tubes were vortexed briefly and 100 mU ST3Gal3 (90  $\mu$ L) were added (total volume 250  $\mu$ L). The tubes were vortexed again and mixed gently for 24 hours at 32 °C. The reactions were stopped by freezing at -80 °C. Novex Tris-Glycine 8-16% 1 mm gels were used for SDS PAGE analysis (Figure 190). Samples (25  $\mu$ L, 25  $\mu$ g) were mixed with 25  $\mu$ L of sample loading buffer and 0.4  $\mu$ L of  $\beta$ -mercaptoethanol and heated for 6 minutes at 85 °C. Gels were run using standard conditions and stained with Colloidal Blue Stain. IEF gels were also performed as described above (Figure 191). Samples were also dialyzed against water and analyzed by MALDI-TOF.

**Results.** MALDI was also performed. Native transferrin (78729); asialotransferrin (78197); resialylated transferrin (79626/80703); with SA-PEG 1k (79037 (1); 80961 (2); 82535 (3); 84778 (4)); with SA-PEG 5k (90003 (2); 96117 (3); 96117 (4)); with SA-PEG 10k (100336 (2); 111421 (3); 122510 (4)).

#### 43. Transferrin-GDNF

This example sets forth the procedures for the glycoconjugation of proteins, and in particular, transferrin is glycoconjugated to GDNF. Transferrin-SA-Linker-Gal-UDP is prepared from transferrin. The galactose residue is removed from GDNF glycans, and

5 Transferrin-SA-Linker-Gal-UDP is conjugated to GDNF glycans using a galactosyltransferase.

**Preparation of agalacto-GDNF.** GDNF produced in NSO cells (NSO murine myeloma cells) is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is incubated with 300 mU/mL beta-galactosidase-agarose conjugate for 16 hours at 10 32°C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The supernatant is dialyzed at 4 °C against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated 15 using a Centricon Plus 20 centrifugal filter and stored at -20 °C. The conditions for the IEF gel are run according to the procedures and reagents provided by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Transferrin-SA-Linker-Gal-UDP.** Asialo-transferrin is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is 20 incubated with CMP-sialic acid-linker-Gal-UDP (molar amount to add 1 molar equivalent of nucleotide sugar to transferrin) and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid, a small aliquot of the reaction has <sup>14</sup>C-SA-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label 25 incorporation into the peptide is quantitated using an in-line radiation detector.

The solution is incubated with 5 mM CMP-sialic acid and 0.1 U/mL of ST3Gal3 (to cap any unreacted transferrin glycans) at 32°C for 2 days. The incorporation into the peptide is quantitated using an in-line UV detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting 30 fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE

and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Transferrin-SA-Linker-Gal-GDNF.** The transferrin-SA-Linker-Gal-UDP prepared as described above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL agalacto-GDNF and 0.1 U/mL of galactosyltransferase at 32°C for 2 days. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the solution is incubated with 5 mM UDP-Gal and 0.1 U/mL of galactosyltransferase (to cap any unreacted transferrin glycans) at 32°C for 2 days followed by addition of 5 mM CMP-SA and 0.1 U/mL of ST3Gal3. After 2 additional days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed:

1. An EPO peptide comprising one or more glycans, having a glycoconjugate molecule covalently attached to said peptide.
2. The EPO peptide of claim 1, wherein said one or more glycans is a  
5 monoantennary glycan.
3. The EPO peptide of claim 1, wherein said one or more glycans is a biantennary glycan.
4. The EPO peptide of claim 1, wherein said one or more glycans is a triantennary glycan.
- 10 5. The EPO peptide of claim 1, wherein said one or more glycans is at least a triantennary glycan.
6. The EPO peptide of claim 1, wherein said one or more glycans comprises at least two glycans comprising a mixture of mono or multiantennary glycans.
7. The EPO peptide of claim 1, wherein said one or more glycans is selected  
15 from an N-linked glycan and an O-linked glycan.
8. The EPO peptide of claim 1, wherein said one or more glycans is at least two glycans selected from an N-linked and an O-linked glycan.
9. The EPO peptide of claim 1, wherein said peptide is expressed in a cell selected from the group consisting of a prokaryotic cell and a eukaryotic cell.
- 20 10. The EPO peptide of claim 9, wherein said eukaryotic cell is selected from the group consisting of a mammalian cell, an insect cell and a fungal cell.
11. The EPO peptide of claim 10, wherein said fungal cell is a yeast cell.
12. A glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to said glycan,

wherein said poly(ethylene glycol) molecule is added to said EPO peptide using a glycosyltransferase.

13. The glycoPEGylated EPO peptide of claim 12, comprising at least one  
5 mono-antennary glycan.

14. The glycoPEGylated EPO peptide of claim 12, wherein all of said glycans are N-linked and are mono-antennary.

10 15. The glycoPEGylated EPO peptide of claim 12, wherein all of said glycans are N-linked and at least one of said glycans comprise said poly(ethylene glycol).

16. The glycoPEGylated EPO peptide of claim 15, wherein more than one of said glycans comprises said poly(ethylene glycol).

15 17. The glycoPEGylated EPO peptide of claim 12, wherein all of said glycans are N-linked and all of said glycans comprise said poly(ethylene glycol).

18. The glycoPEGylated EPO peptide of claim 12, comprising at least three  
20 mono-antennary glycans having said poly(ethylene glycol) covalently attached thereto.

19. A glycoPEGylated EPO peptide, wherein said EPO peptide comprises three or more glycans.

25 20. The glycoPEGylated EPO peptide of claim 9, wherein at least one of said glycans comprises said poly(ethylene glycol) covalently attached thereto.

21. The glycoPEGylated EPO peptide of claim 18, wherein more than one of said glycans comprises said poly(ethylene glycol) covalently attached thereto.

30



22. The glycoPEGylated EPO peptide of claim 18, wherein all of said glycans comprise said poly(ethylene glycol) covalently attached thereto.

23. The glycoPEGylated EPO peptide of claim 12 wherein said poly(ethylene glycol) is linked to at least one sugar moiety selected from the group consisting of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal) and a sialic acid (SA).

24. The glycoPEGylated EPO peptide of claim 23, wherein said sialic acid is N-acetylneuraminic acid.

25. The glycoPEGylated EPO peptide of claim 12, wherein said EPO peptide does not comprise an O-linked glycan.

26. The glycoPEGylated EPO peptide of claim 12 wherein said EPO peptide comprises at least one O-linked glycan.

27. The glycoPEGylated EPO peptide of claim 26, wherein said O-linked peptide comprises said poly(ethylene glycol) covalently attached thereto.

28. The glycoPEGylated EPO peptide of claim 27, wherein said EPO peptide is recombinantly expressed in a cell.

29. The glycoPEGylated EPO peptide of claim 28, wherein said cell is selected from the group consisting of an insect cell, a fungal cell and a mammalian cell.

30. The glycoPEGylated EPO peptide of claim 29, wherein said fungal cell is a yeast cell.

31. The glycoPEGylated EPO peptide of claim 29, wherein said cell is an insect cell.

32. The glycoPEGylated EPO peptide of claim 29, wherein said cell is a yeast cell.

33. The glycoPEGylated EPO peptide of claim 29, wherein said cell is a mammalian cell.

34. The glycoPEGylated EPO peptide of claim 33, wherein said mammalian cell is a CHO cell.

35. The glycoPEGylated EPO peptide of claim 12, wherein said poly(ethylene glycol) has a molecular weight selected from the group consisting of about 1 kDa, 2 kDa, 5 kDa, 10 kDa, 20 kDa, 30 kDa and 40 kDa.

36. The glycoPEGylated EPO peptide of claim 35, wherein said poly(ethylene glycol) has a molecular weight of 20 kDa.

37. The glycoPEGylated EPO peptide of claim 12, wherein said EPO peptide is selected from the group consisting of a naturally occurring EPO peptide and a mutated EPO peptide.

38. The glycoPEGylated EPO peptide of claim 37, wherein said mutated EPO peptide comprises the amino acid sequence of SEQ ID NO:73 having at least one mutation selected from the group consisting of Arg<sup>139</sup> to Ala<sup>139</sup>, Arg<sup>143</sup> to Ala<sup>143</sup> and Lys<sup>154</sup> to Ala<sup>154</sup>.

39. A method of making a glycoPEGylated EPO peptide, said method comprising the step of:

(a) contacting an EPO peptide with a mixture comprising a nucleotide sugar covalently linked to poly(ethylene glycol) and a glycosyltransferase under conditions sufficient to transfer said poly(ethylene glycol) to said EPO peptide.

40. The method of claim 39, wherein the sugar of said nucleotide sugar is selected from the group consisting of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal) and a sialic acid (SA).

5                   41. The method of claim 40, wherein said sialic acid is N-acetylneuraminic acid (NAN).

                  42. The method of claim 39, wherein said poly(ethylene glycol) has a molecular weight selected from the group consisting of about 1 kDa, 2 kDa, 5 kDa, 10 kDa,  
10   20 kDa, 30 kDa and 40 kDa.

                  43. The method of claim 42, wherein said poly(ethylene glycol) has a molecular weight of 20 kDa.

15                  44. The method of claim 39, wherein said EPO peptide is recombinantly expressed in a cell.

                  45. The method of claim 44, wherein said cell is selected from the group consisting of an insect cell, a fungal cell and a mammalian cell.

20                  46. The method of claim 45, wherein said cell is an insect cell.

                  47. The method of claim 45, wherein said cell is a yeast cell.

25                  48. The method of claim 45, wherein said cell is a mammalian cell.

                  49. The method of claim 48, wherein said mammalian cell is a CHO cell.

                  50. The method of claim 39, wherein said EPO peptide is selected from the  
30   group consisting of a naturally occurring EPO peptide and a mutated EPO peptide.

51. The method of claim 50, wherein said mature EPO peptide has the sequence of SEQ ID NO:73.

52. The method of claim 50, wherein said mutated EPO peptide comprises the amino acid sequence of SEQ ID NO: 73 having at least one mutation selected from the group consisting of Arg<sup>139</sup> to Ala<sup>139</sup>, Arg<sup>143</sup> to Ala<sup>143</sup> and Lys<sup>154</sup> to Ala<sup>154</sup>.

53. The method of claim 39, wherein before step (a):

(b) contacting said EPO peptide with a mixture comprising a nucleotide-N-acetylglucosamine (GlcNAc) molecule and an N-acetylglucosamine transferase (GnT) for which the nucleotide-GlcNAc is a substrate under conditions sufficient to form a bond between said GlcNAc and said EPO, wherein said GnT is selected from the group consisting of GnT I, GnT II, GnT III, GnT IV, GnT V and GnT VI.

54. The method of claim 53, wherein said mixture comprises one GnT selected from the group consisting of GnT I, GnT II, GnT IV, GnT V and GnT VI.

55. The method of claim 54, wherein said GnT is GnT I.

56. The method of claim 54, wherein said GnT is GnT II.

57. The method of claim 39, wherein said glycoPEGylated EPO peptide comprises at least one mono-antennary glycan.

58. The method of claim 39, wherein the sugar of said nucleotide sugar is galactose and said glycosyltransferase is galactosyl transferase I (GalT I).

59. The method of claim 53, wherein before step (a) but after step (b):

(c) contacting said EPO peptide with a mixture comprising a nucleotide galactose (Gal) and galactosyl transferase I (GalT I) under conditions sufficient to transfer galactose to said EPO peptide.

60. The method of claim 39, wherein in step (a), the sugar of said nucleotide sugar is sialic acid and said glycosyltransferase is a sialyltransferase.

5           61. The method of claim 60, wherein said sialic acid is N-acetylneuraminic acid (NAN).

62. The method of claim 60, wherein said sialyltransferase is selected from the group consisting of  $\alpha$ (2,3)sialyltransferase,  $\alpha$ (2,6)sialyltransferase and  
10   (2,8)sialyltransferase.

63. A glycoPEGylated EPO peptide made by the method of claim 39.

64. A glycoPEGylated EPO peptide, said EPO peptide comprising the  
15   sequence of SEQ ID NO:73.

65. A glycoPEGylated EPO peptide, said EPO peptide comprising the sequence of SEQ ID NO:73 and further comprising a mutation in said sequence.

20           66. A method of making a glycoPEGylated EPO peptide, said method comprising the steps of:

          (a) contacting an EPO peptide with a mixture comprising a nucleotide sugar covalently linked to poly(ethylene glycol) and a glycosyltransferase under conditions sufficient to transfer said poly(ethylene glycol) to said EPO peptide, wherein said  
25   glycosyltransferase is a fucosyltransferase.

67. The method of claim 66, wherein said fucosyltransferase is selected from the group consisting of fucosyltransferase I, fucosyltransferase III, fucosyltransferase IV, fucosyltransferase V, fucosyltransferase VI and fucosyltransferase VII.

30           68. A glycoPEGylated EPO peptide made by the method of claim 66.

69. The method of claim 66, wherein said EPO peptide is expressed in a CHO cell.

5           70. A method of treating a mammal having anemia, said method comprising administering to said mammal an EPO peptide having one or more glycans having a glycoconjugate molecule attached to said peptide; wherein said EPO peptide is administered in an amount effective to increase the hematocrit level in said mammal.

10           71. The method of claim 70, wherein said mammal is a human.

          72. A method of providing erythropoietin therapy to a mammal, said method comprising administering an effective amount of a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule  
15 covalently attached to said glycan, wherein said poly(ethylene glycol) molecule is added to said EPO peptide using a glycosyltransferase, wherein said EPO peptide is administered in an amount effective to increase the hematocrit level in said mammal.

          73. The method of claim 72, wherein said mammal is a human.  
20

          74. A method of treating a mammal having anemia, said method comprising administering to said mammal a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to said glycan, wherein said poly(ethylene glycol) molecule is added to said EPO peptide using  
25 a glycosyltransferase, wherein said EPO peptide is administered in an amount effective to increase the hematocrit level in said mammal..

          75. The method of claim 74, wherein said mammal is a human.  
30

76. The method of claim 75, wherein said anemia is associated with chemotherapy.

77. A method of treating a kidney dialysis patient, said method comprising  
5 administering to said patient a glycoPEGylated EPO peptide comprising an EPO peptide and  
at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to said  
glycan, wherein said poly(ethylene glycol) molecule is added to said EPO peptide using a  
glycosyltransferase, wherein said EPO peptide is administered in an amount effective to  
increase the hematocrit level in said patient.

10

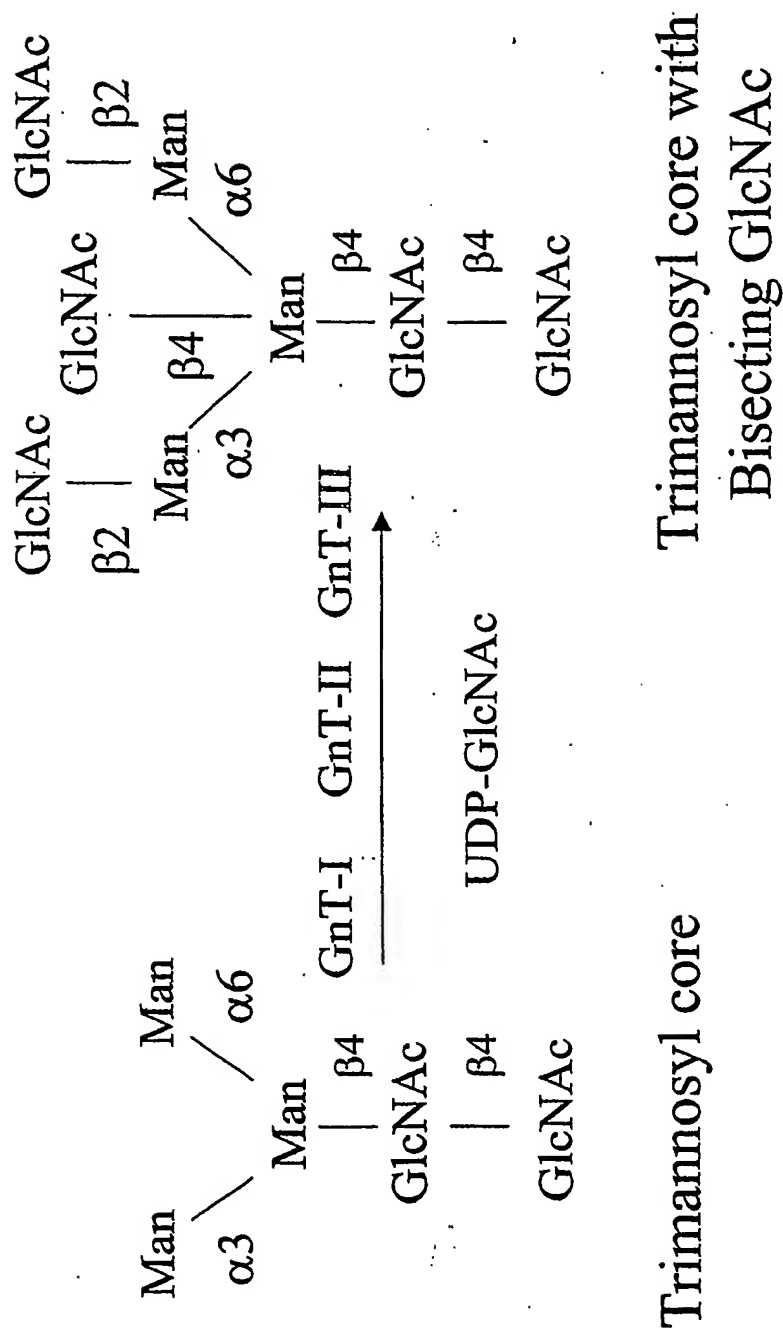


FIG. 1



2/498

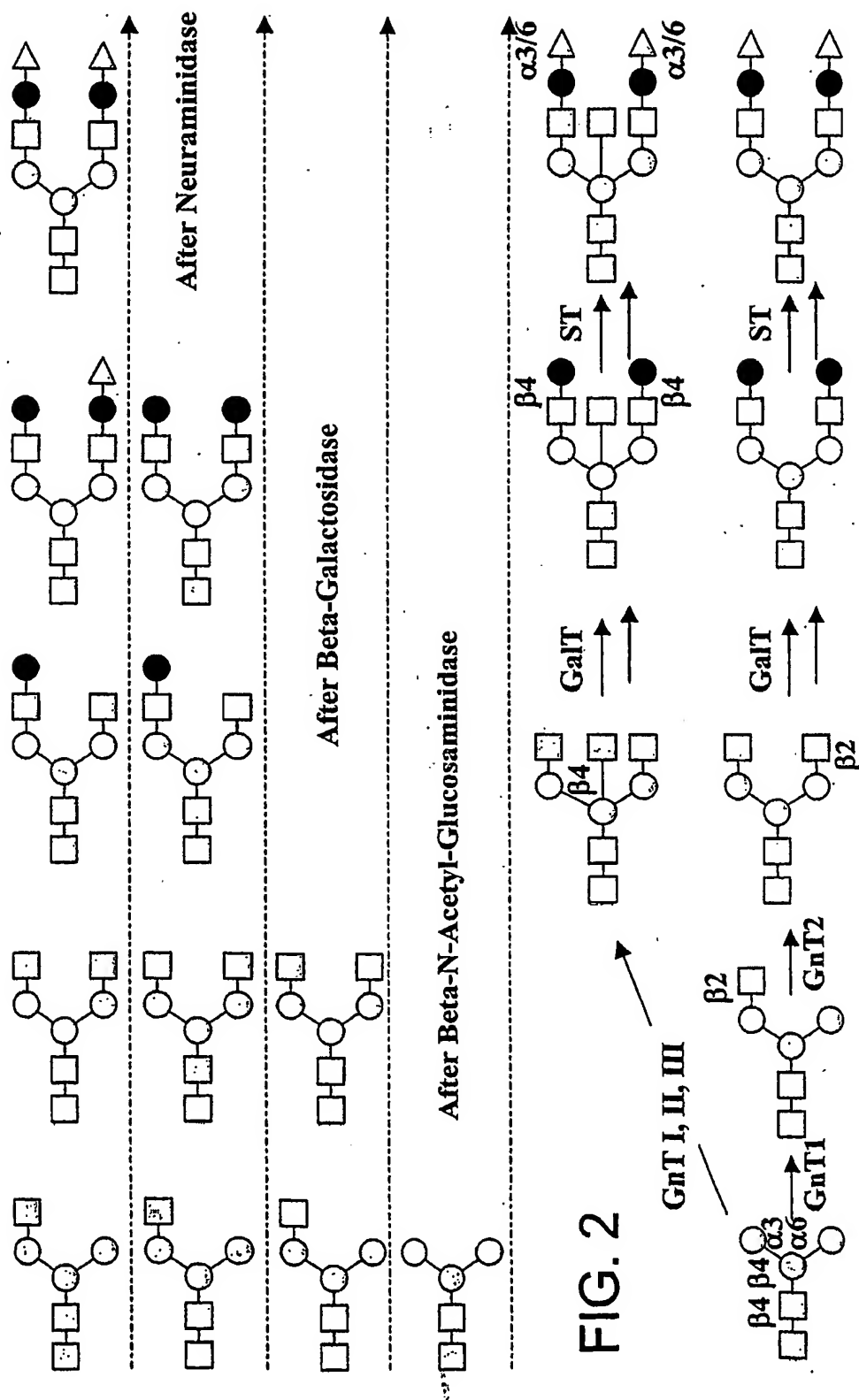


FIG. 2

3/498

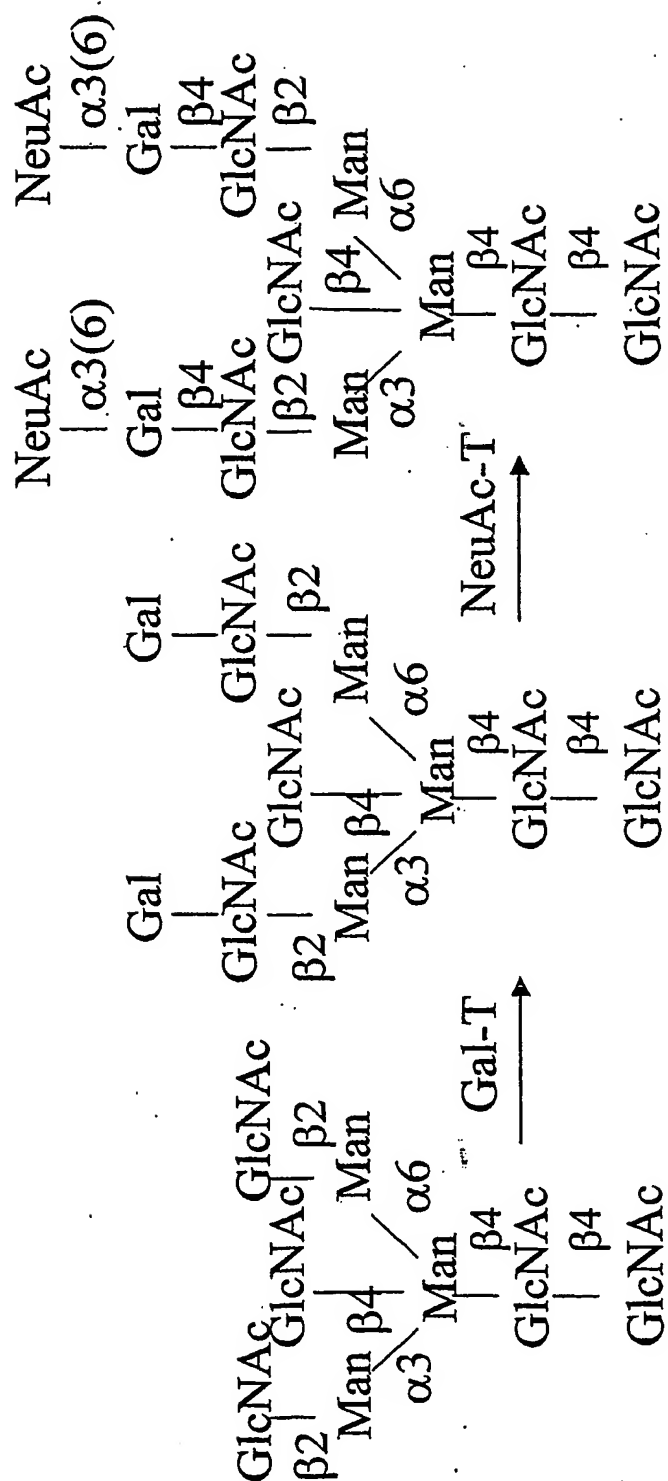


FIG. 3

4/498

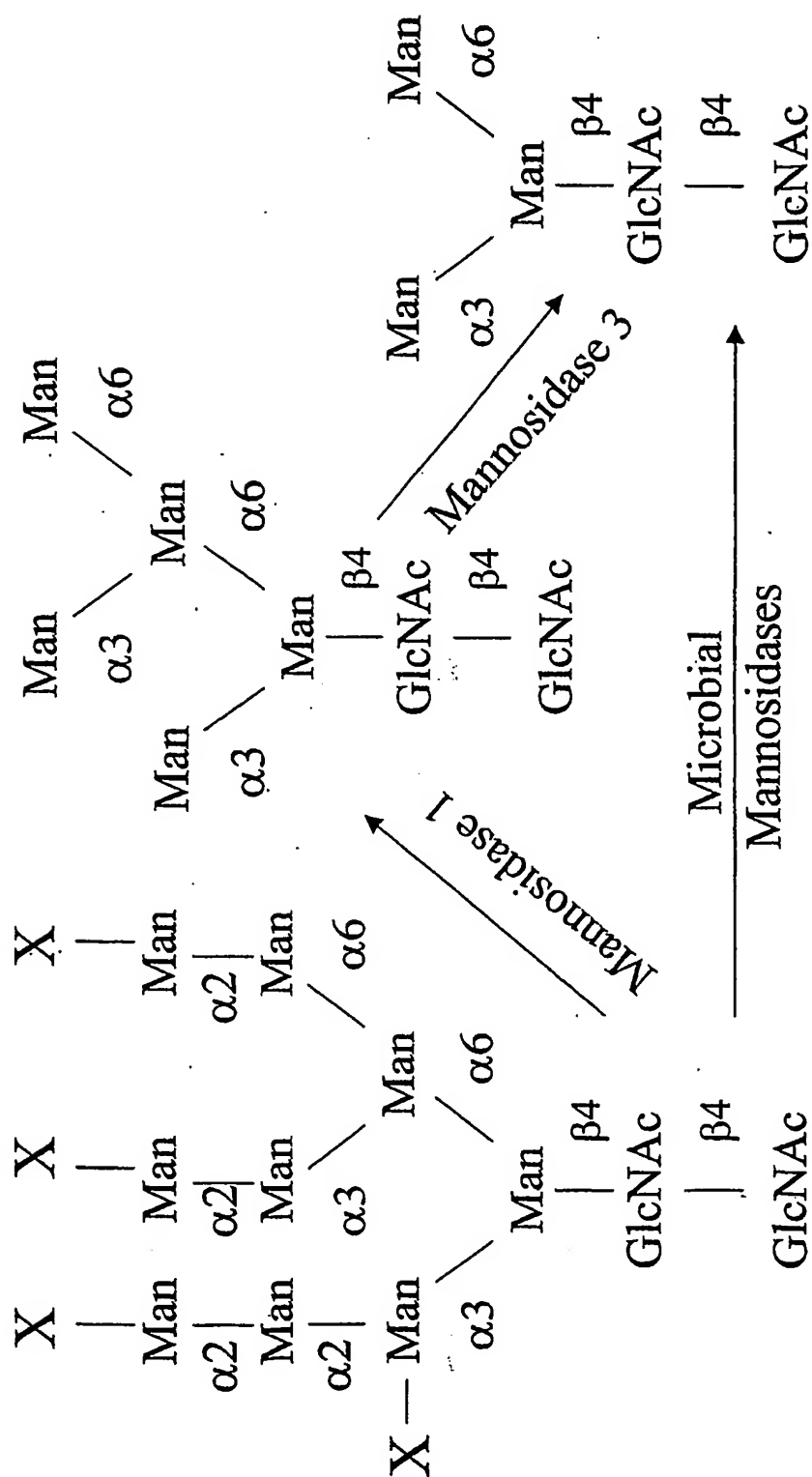
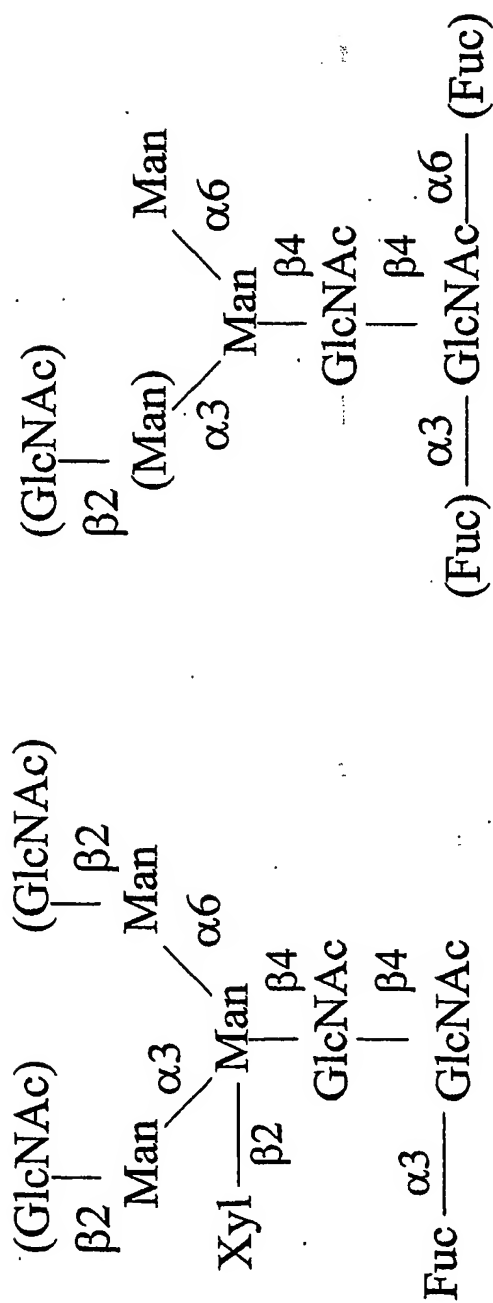
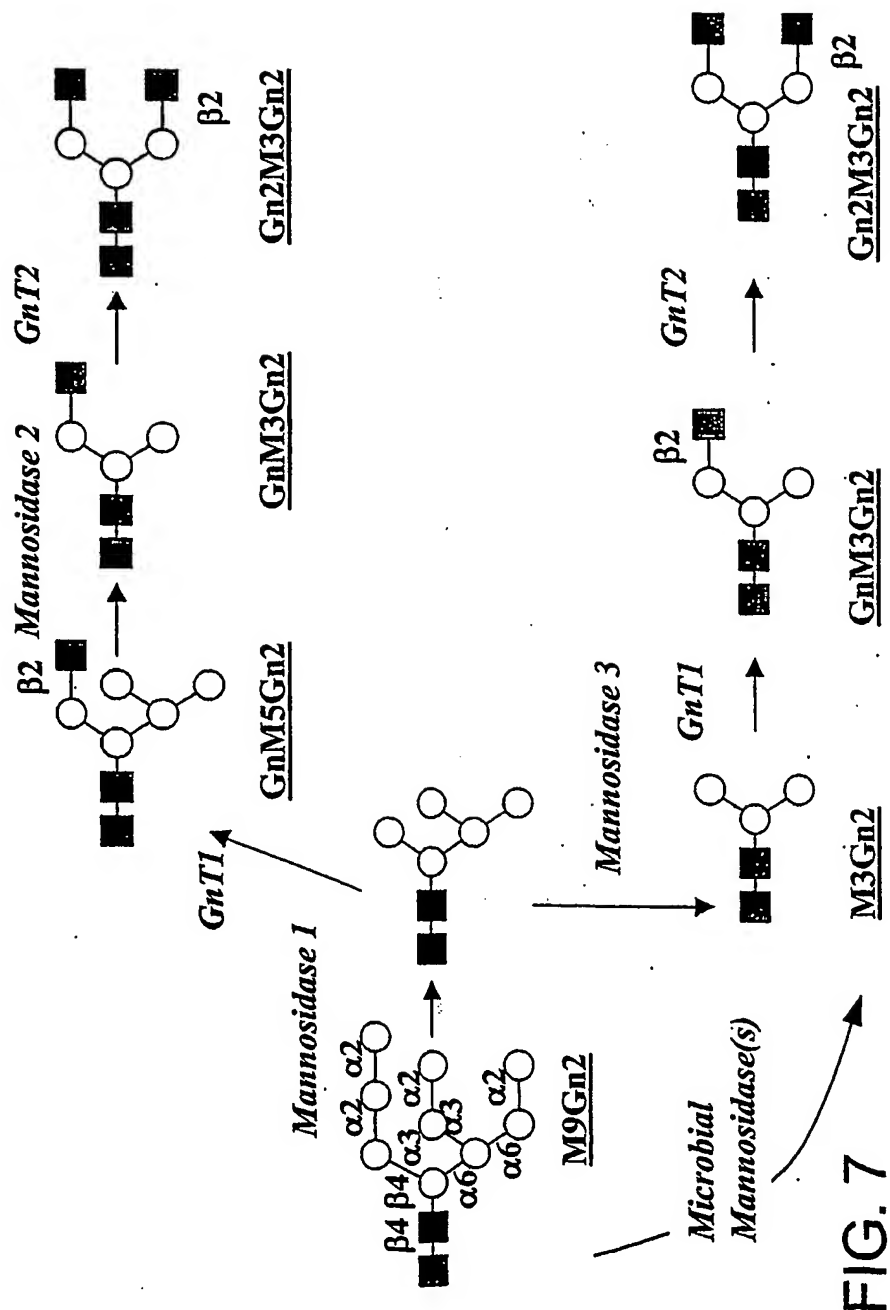


FIG. 4

5/498



6/498



7/498

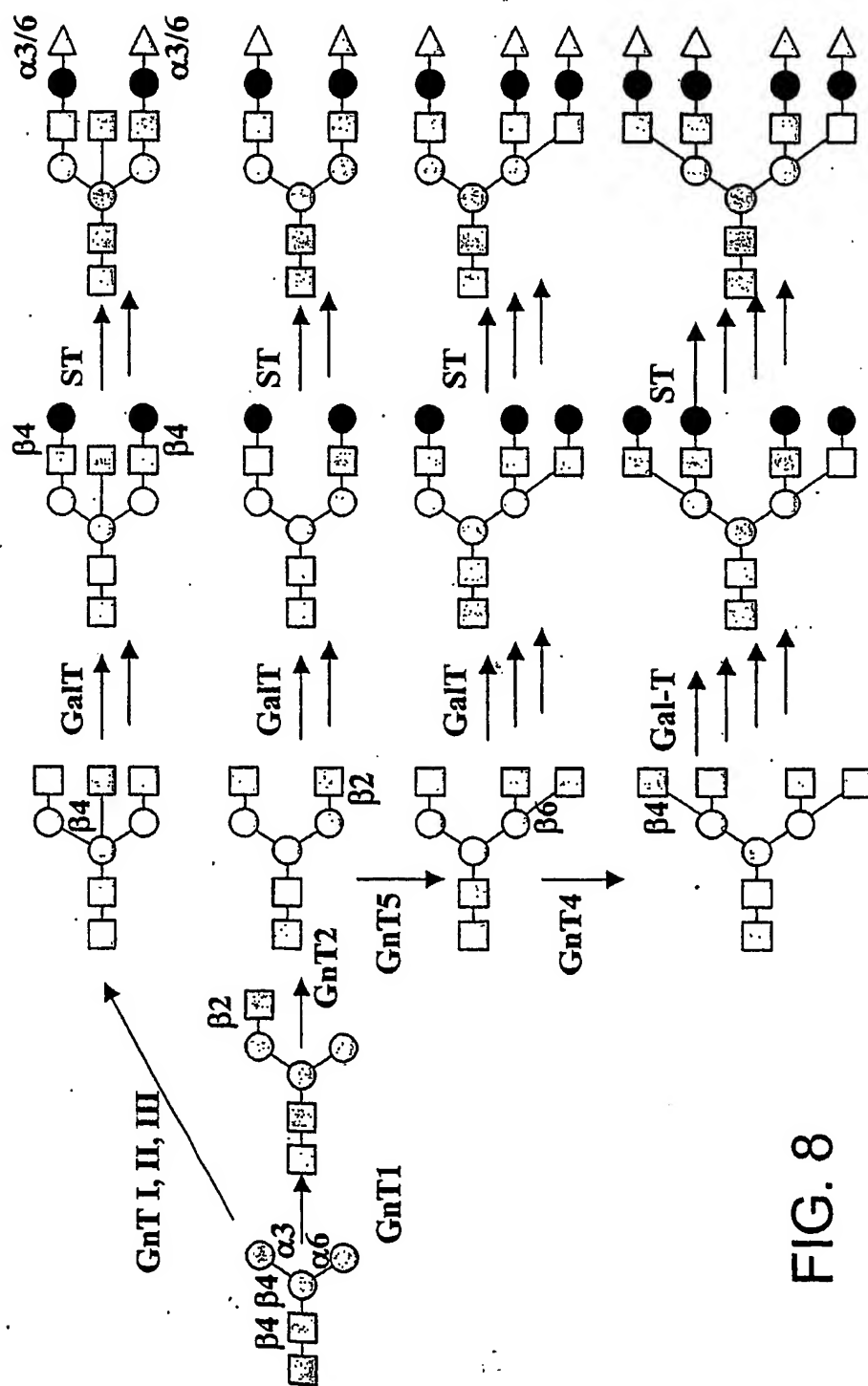


FIG. 8

8/498

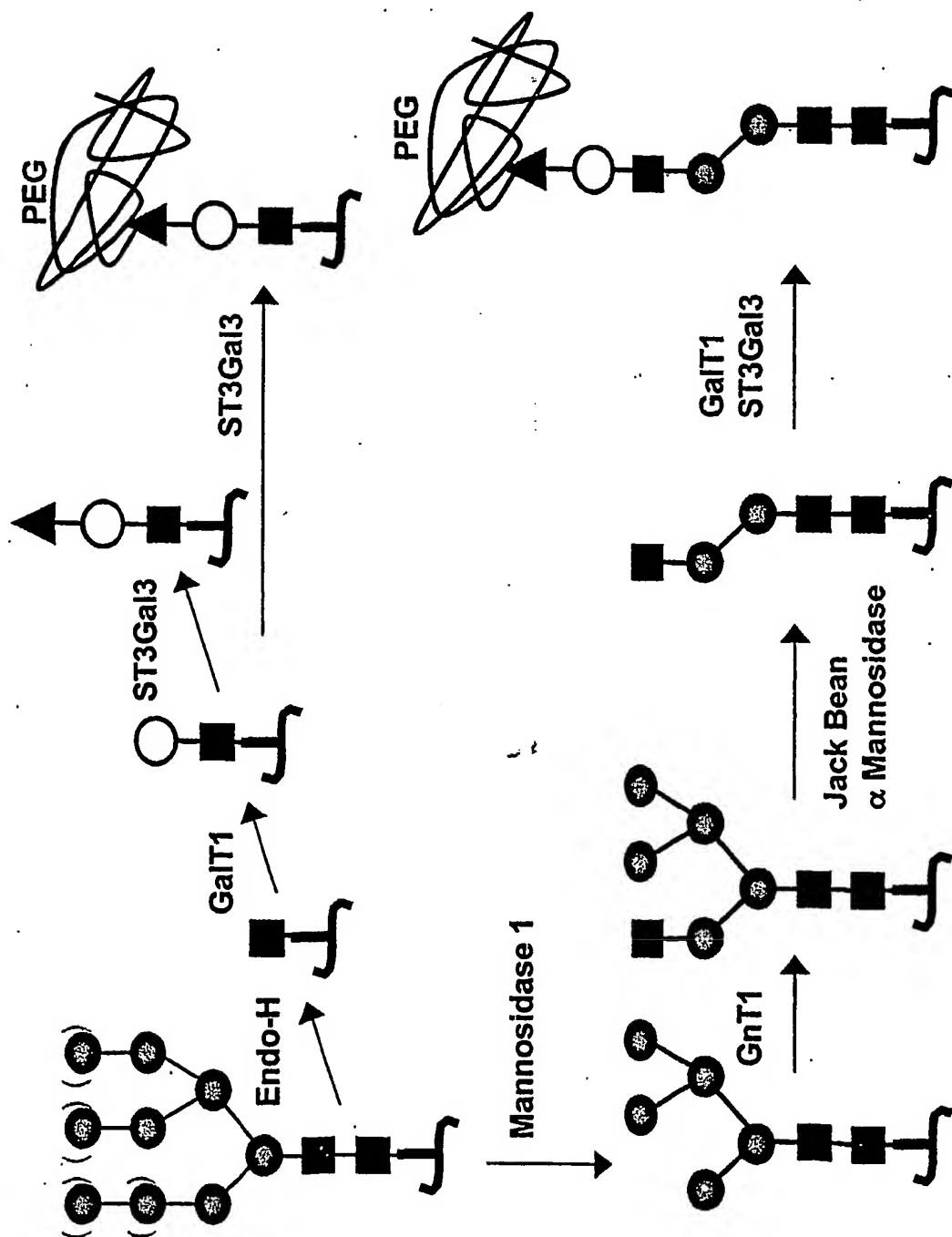


FIG. 9

9/498

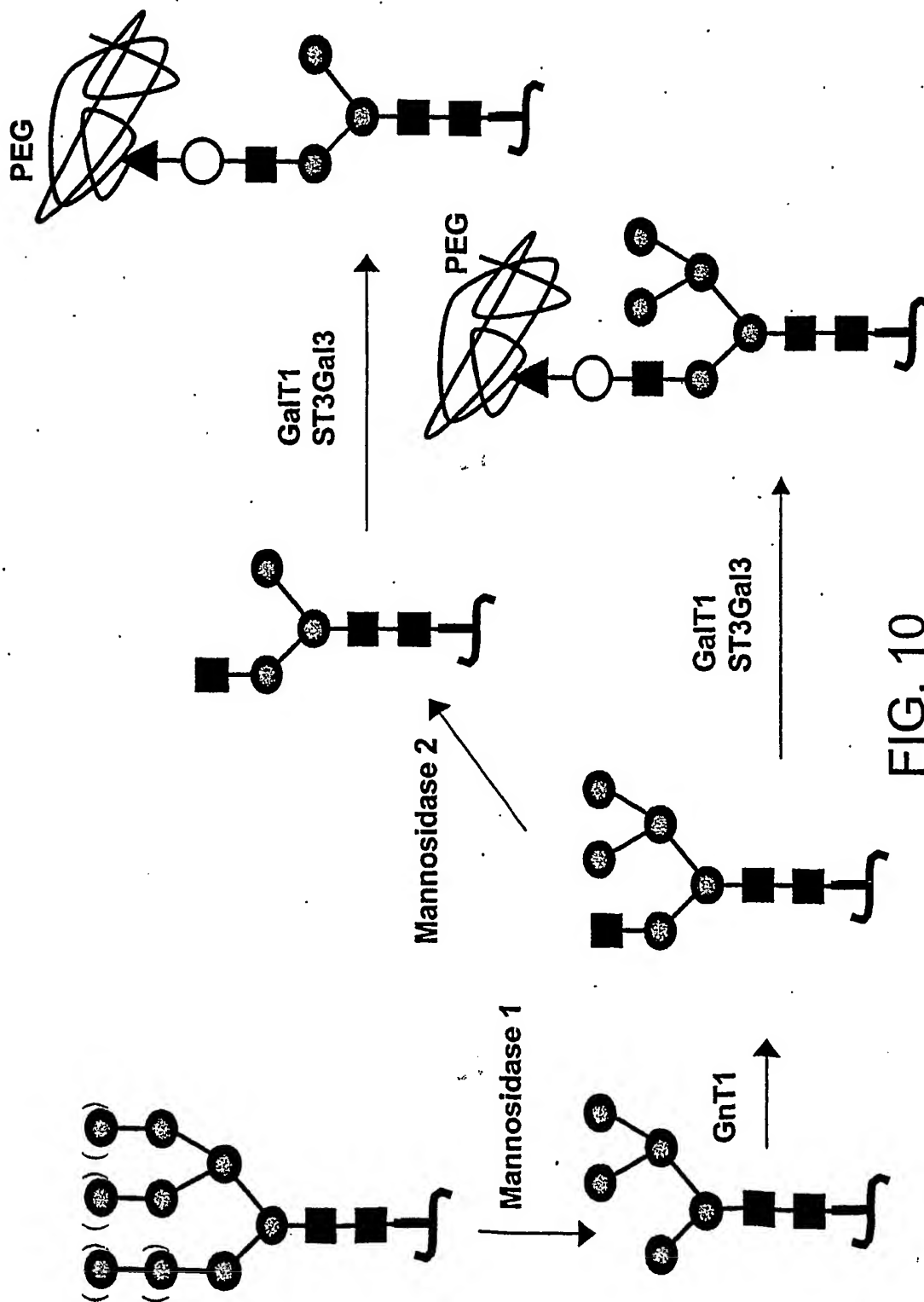


FIG. 10



10/498

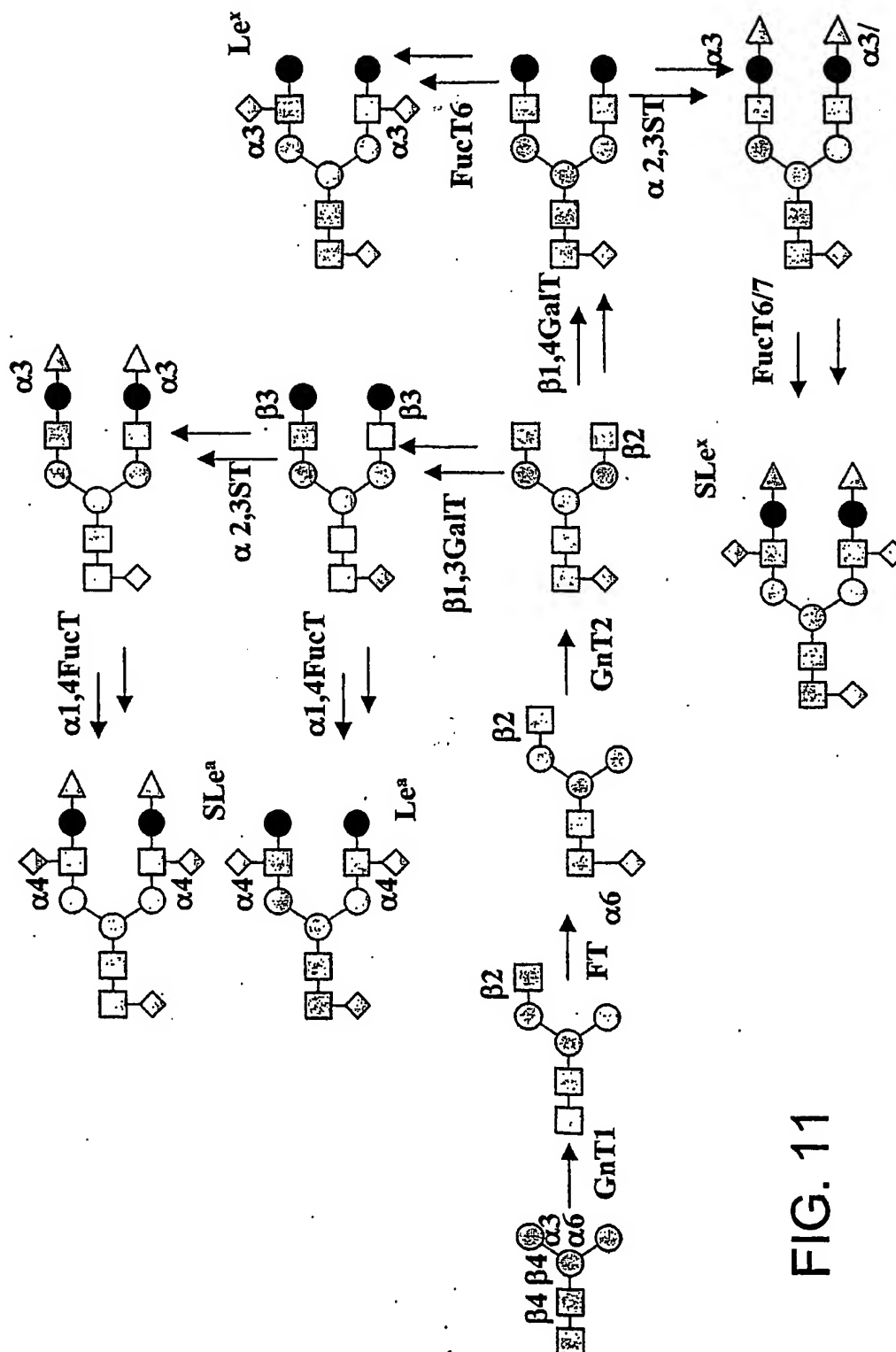
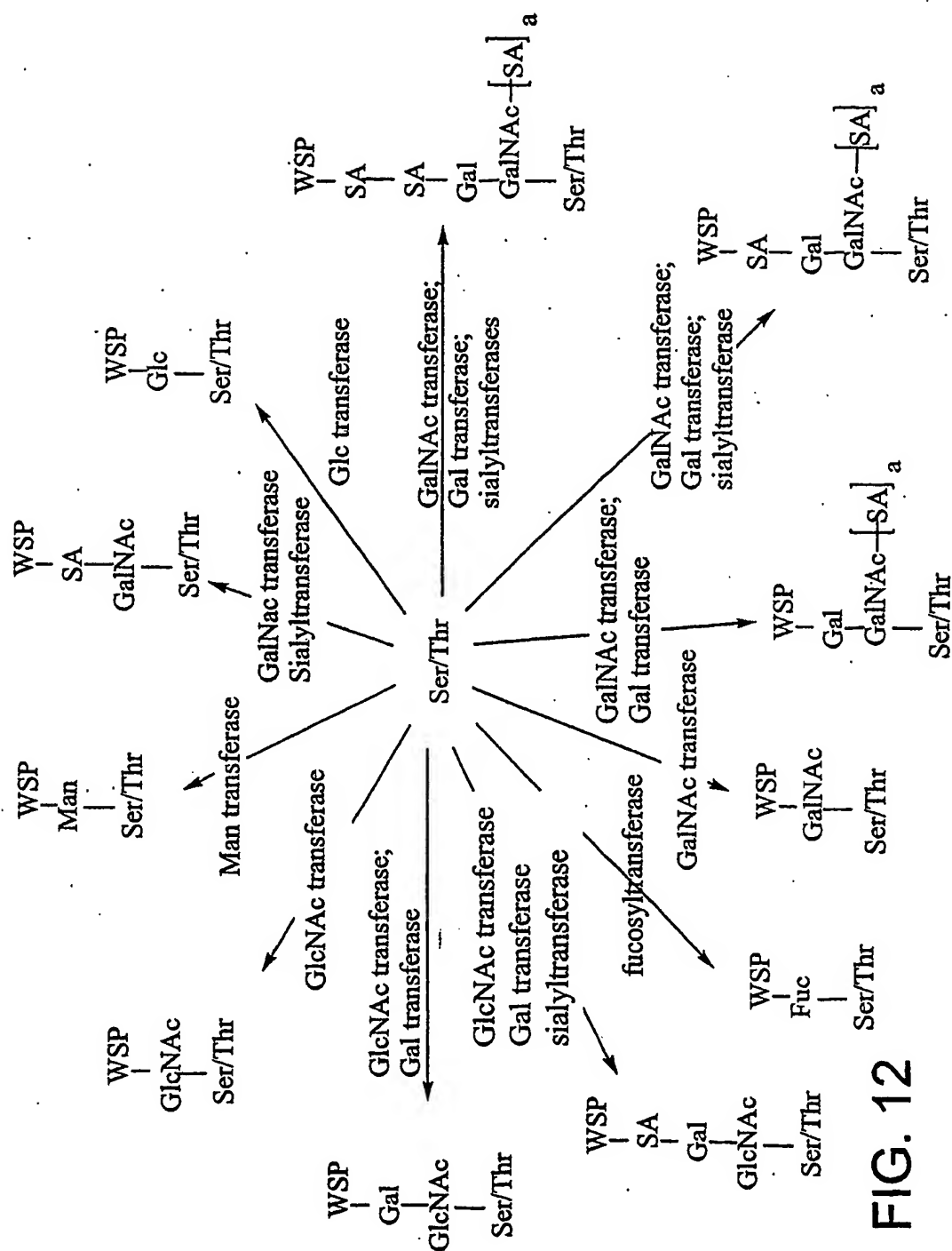


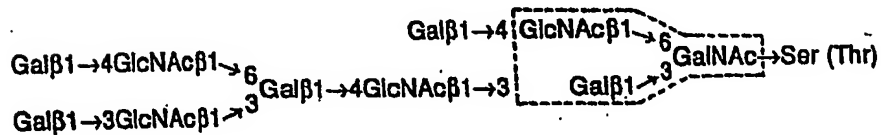
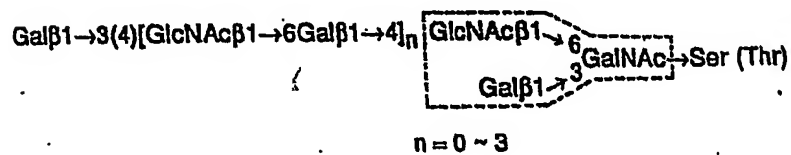
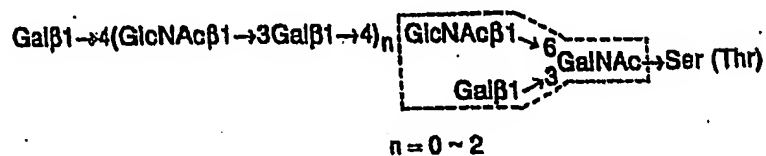
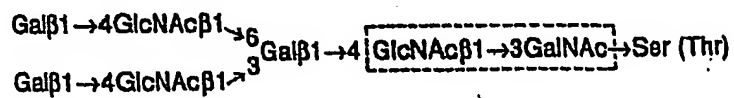
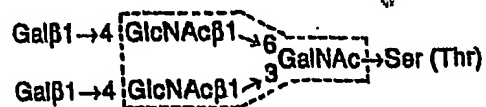
FIG. 11

11/498



**FIG. 12**

12/498

**Core 1****Core 2****Core 3****Core 4****FIG. 13**



14/498

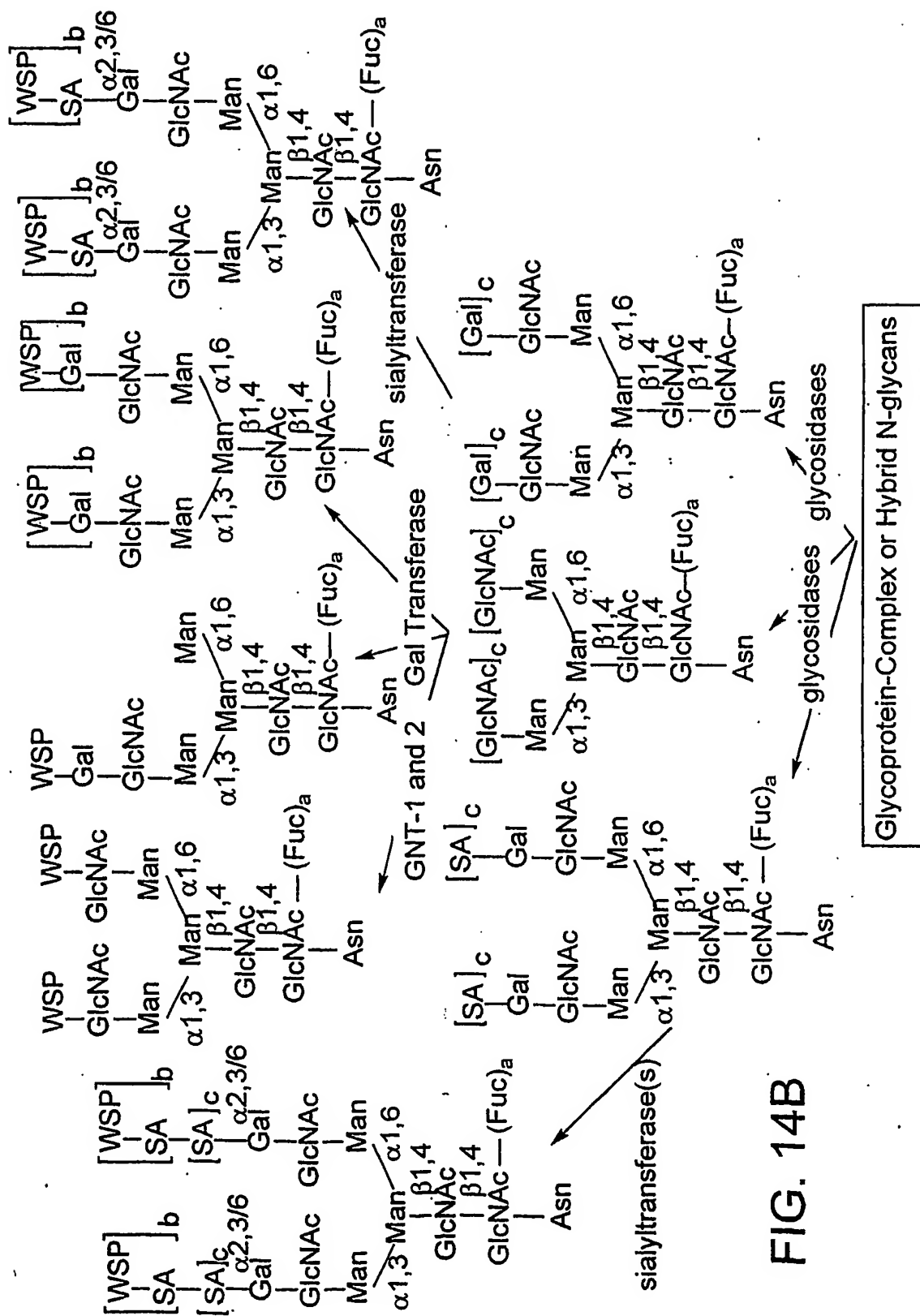


FIG. 14B

15/498

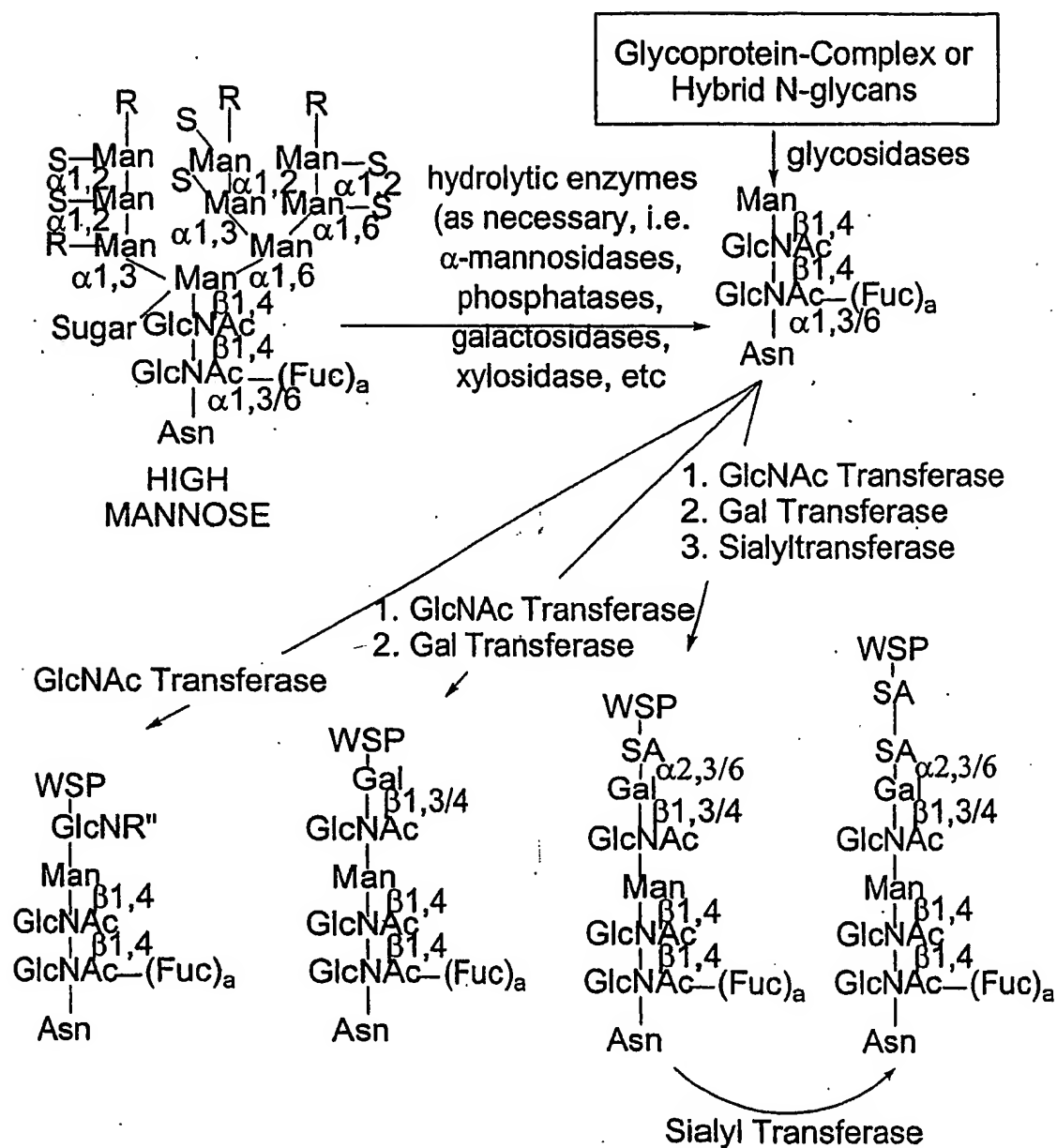


FIG. 15

16/498

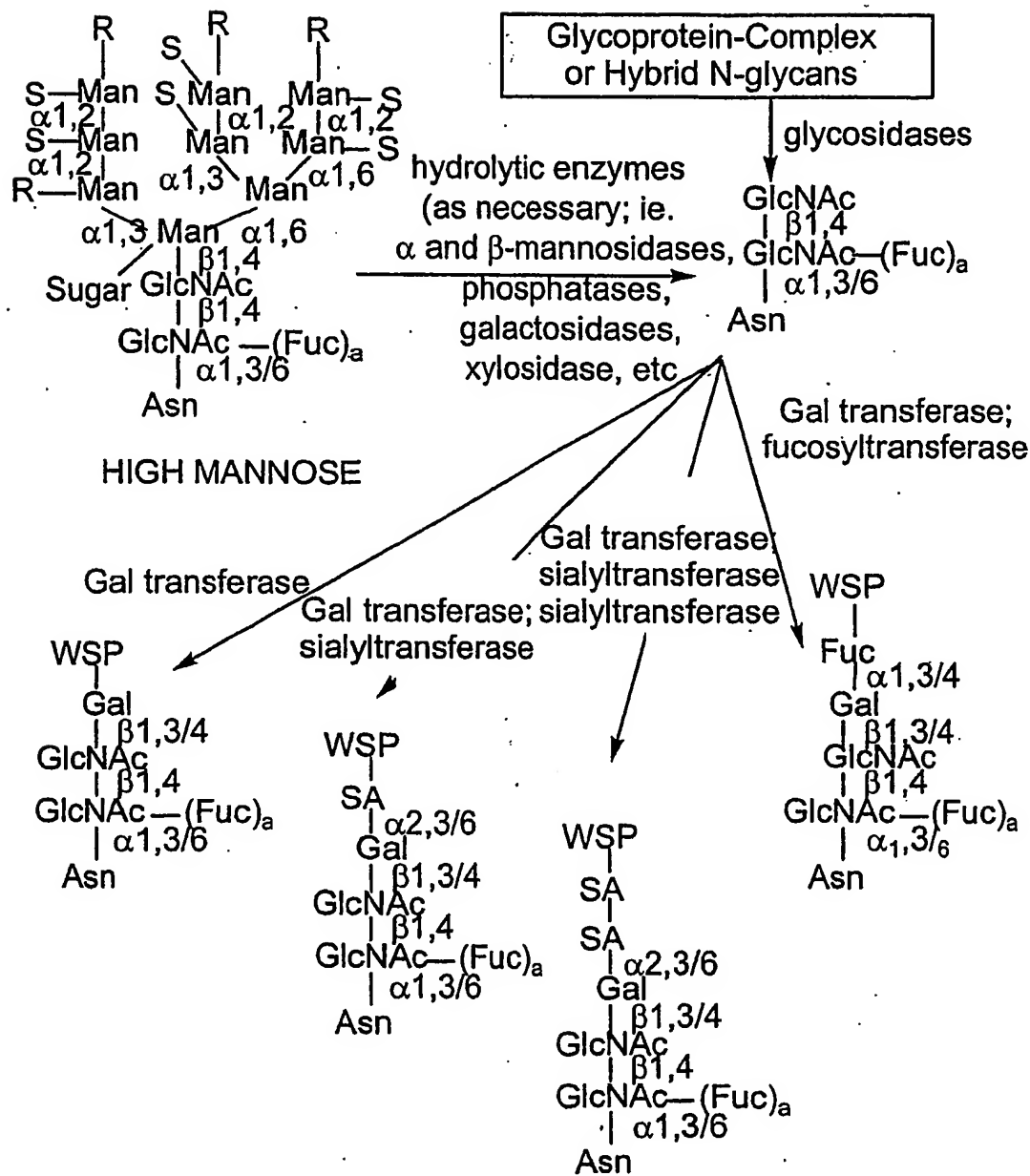


FIG. 16

17/498

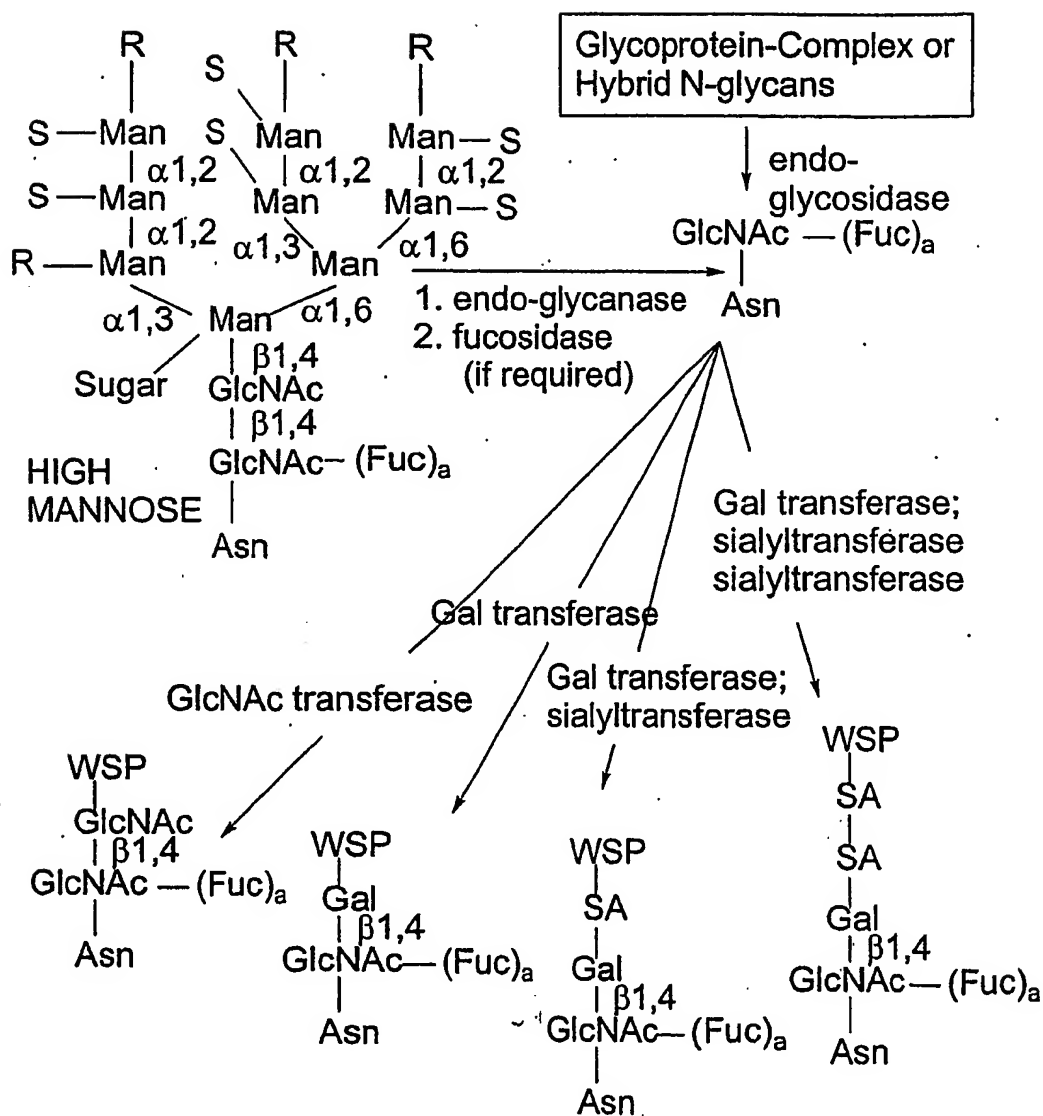


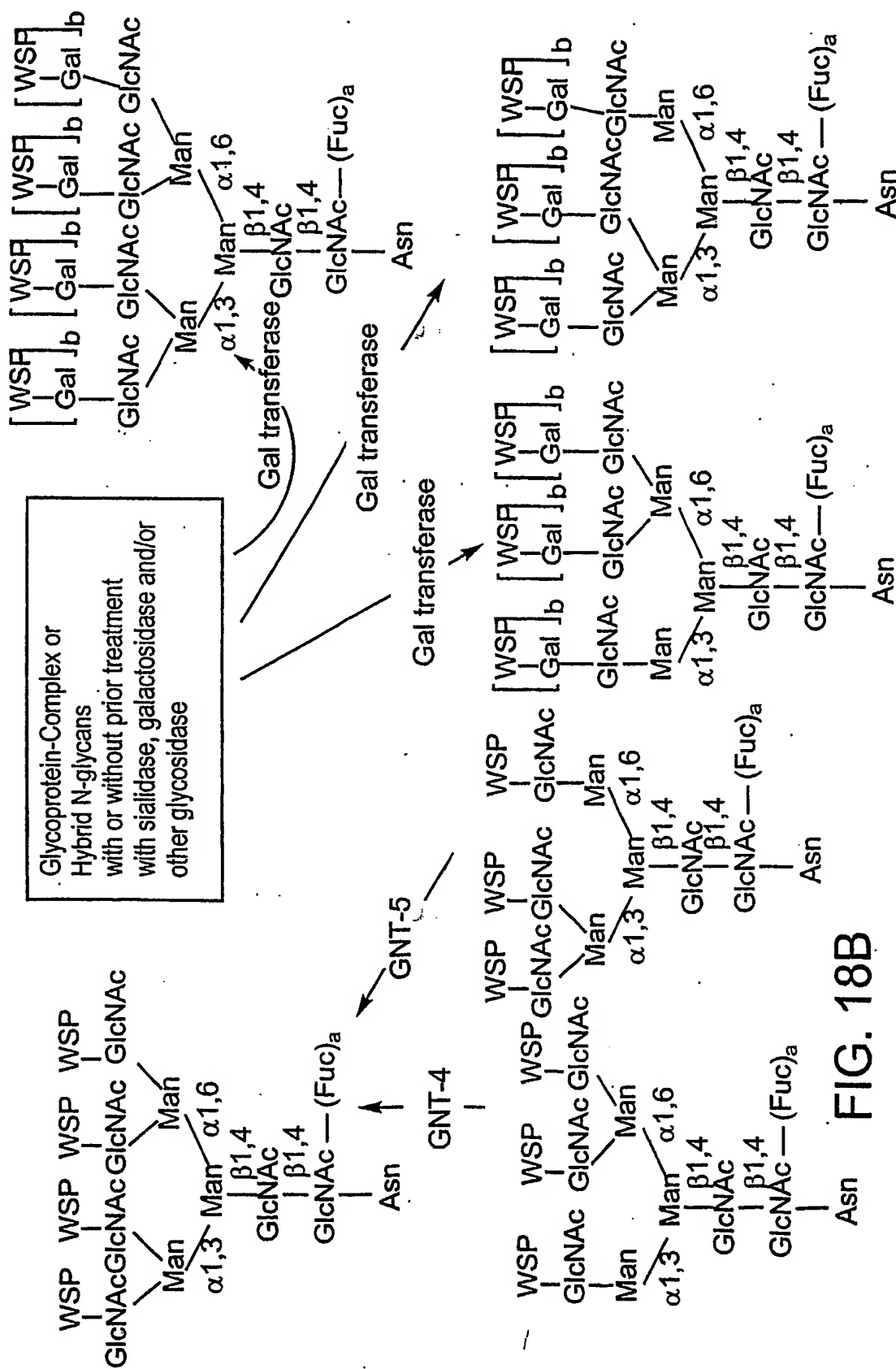
FIG. 17



**FIG. 18A**

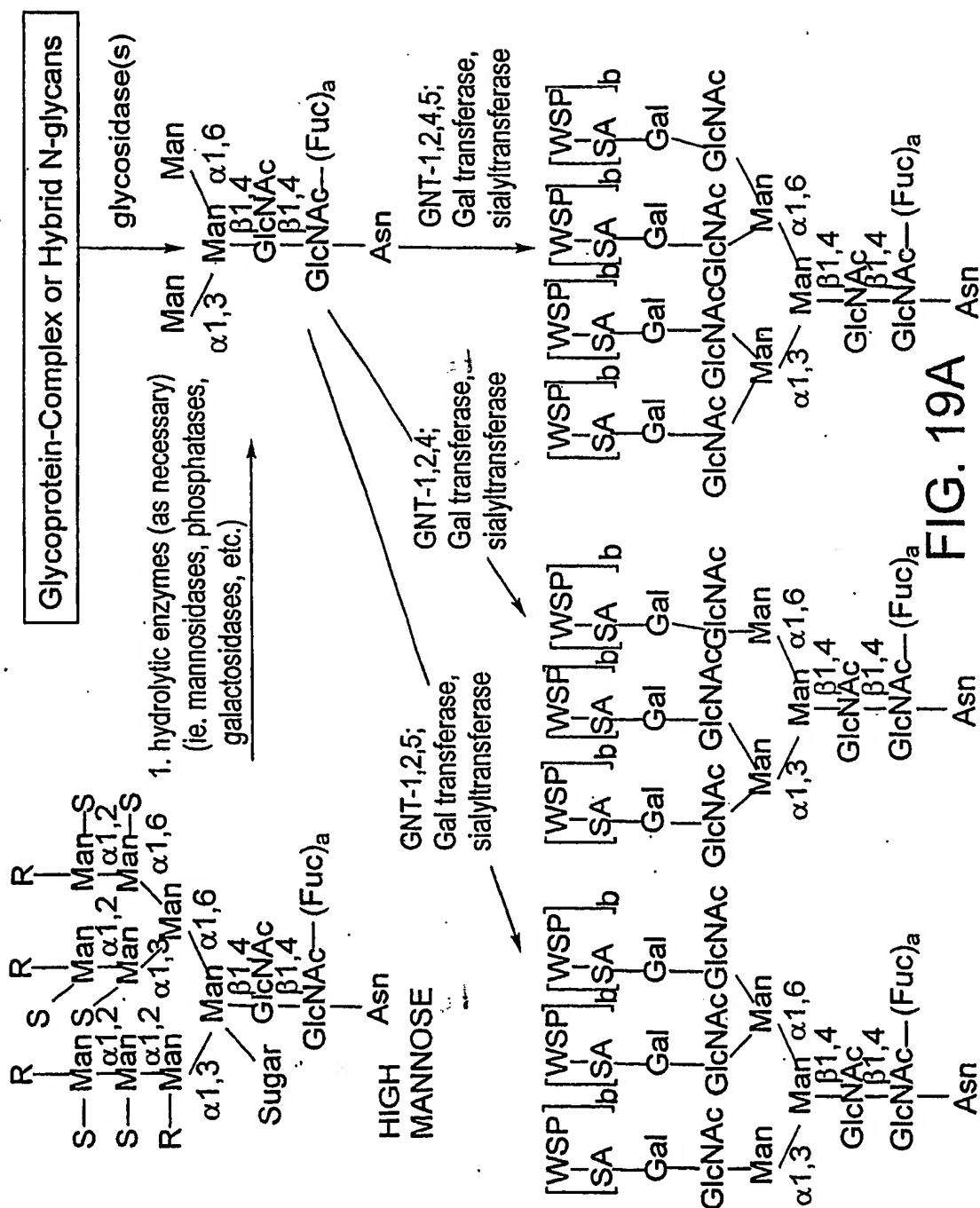
FIG. 18A

19/498

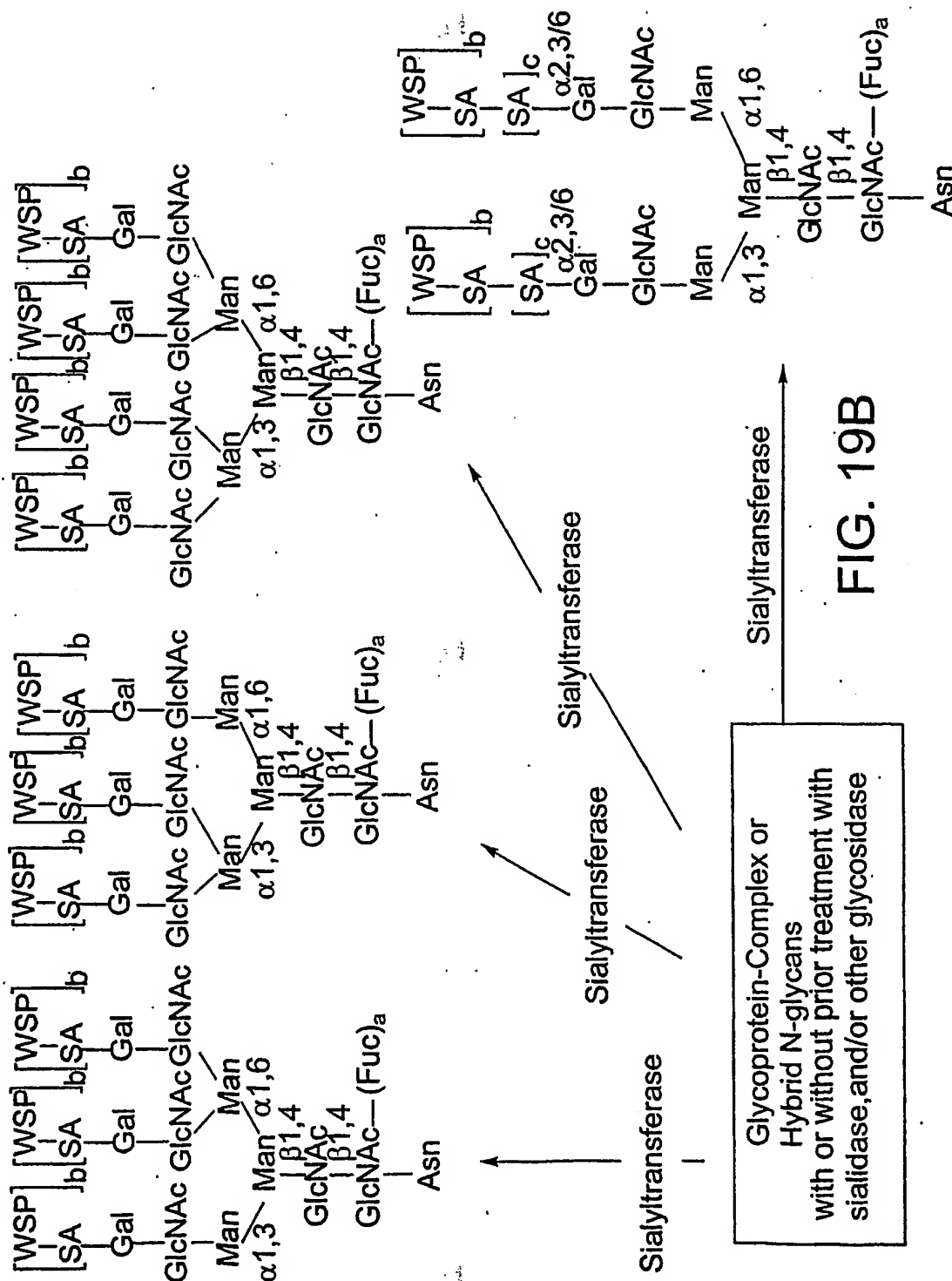


**FIG. 18B**

20/498



21/498



22/498

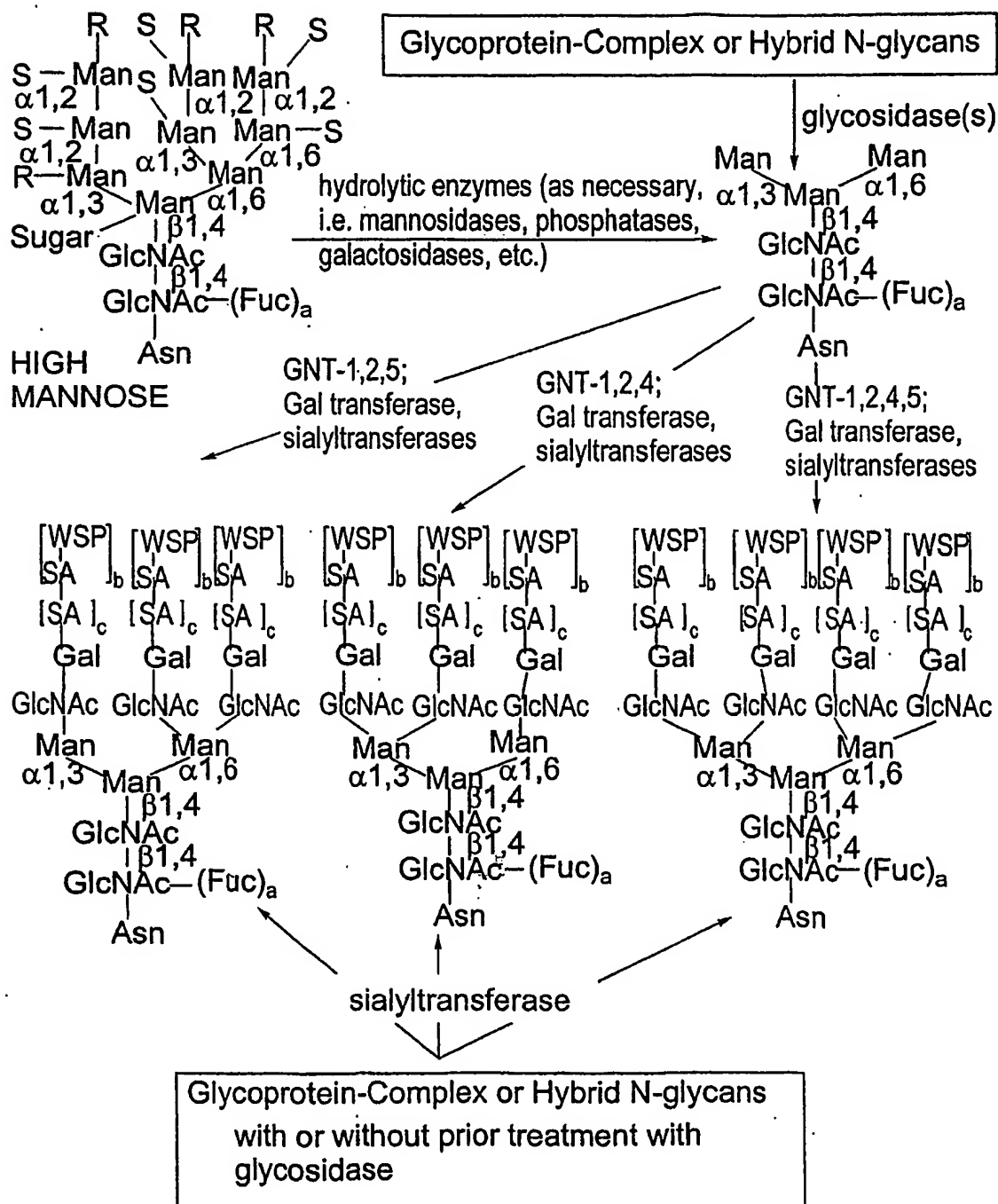
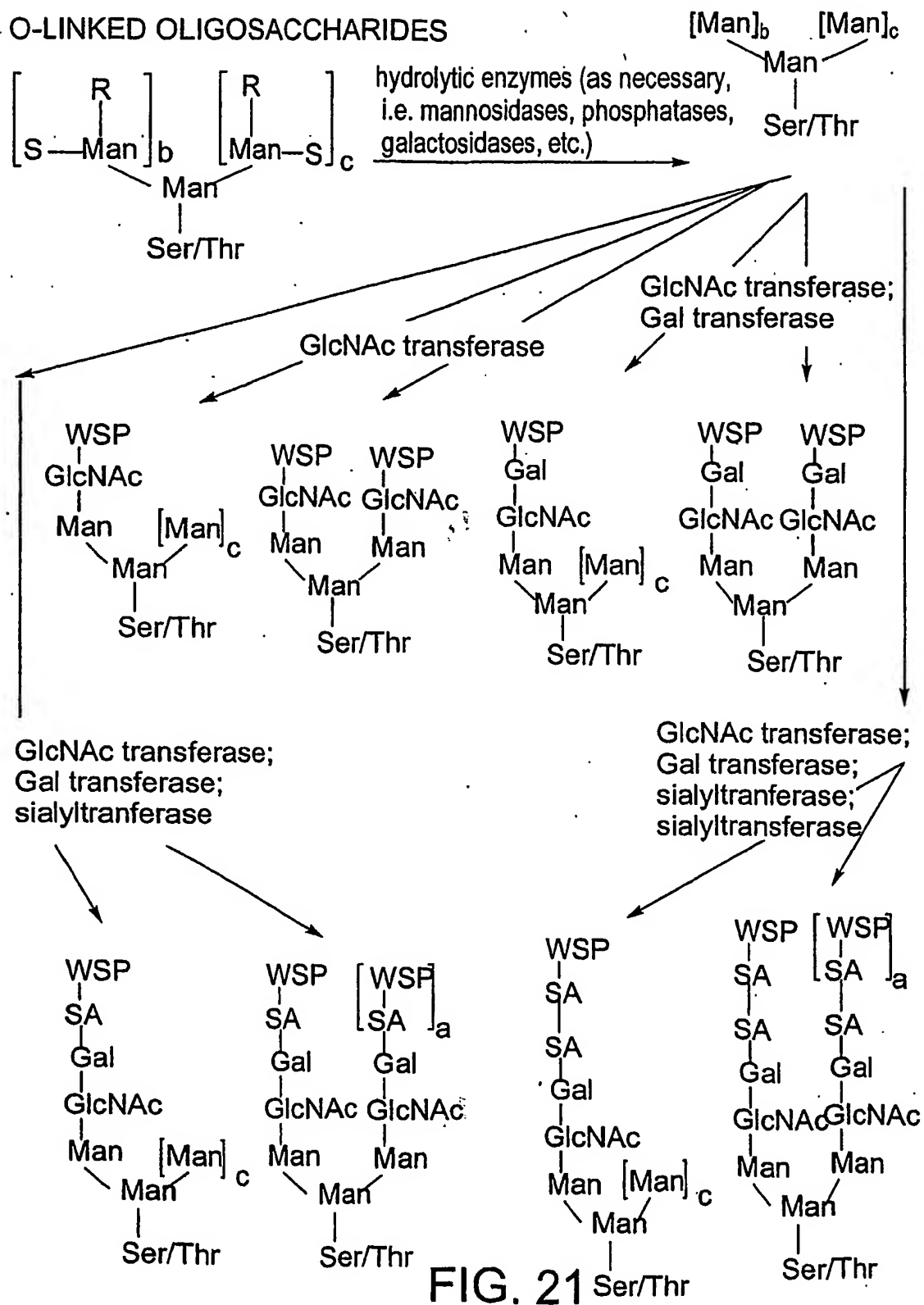


FIG. 20

23/498

## O-LINKED OLIGOSACCHARIDES



24/498

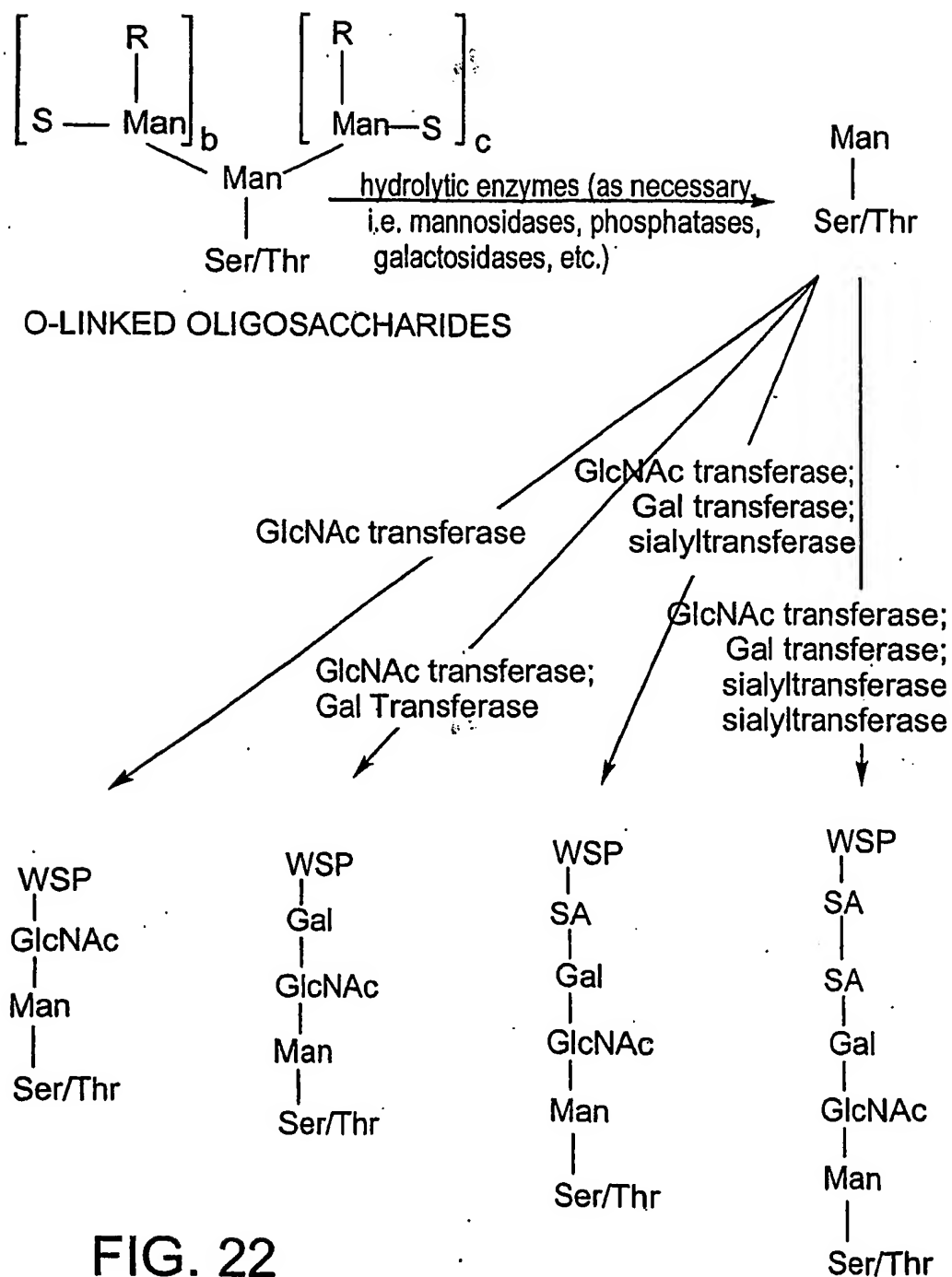
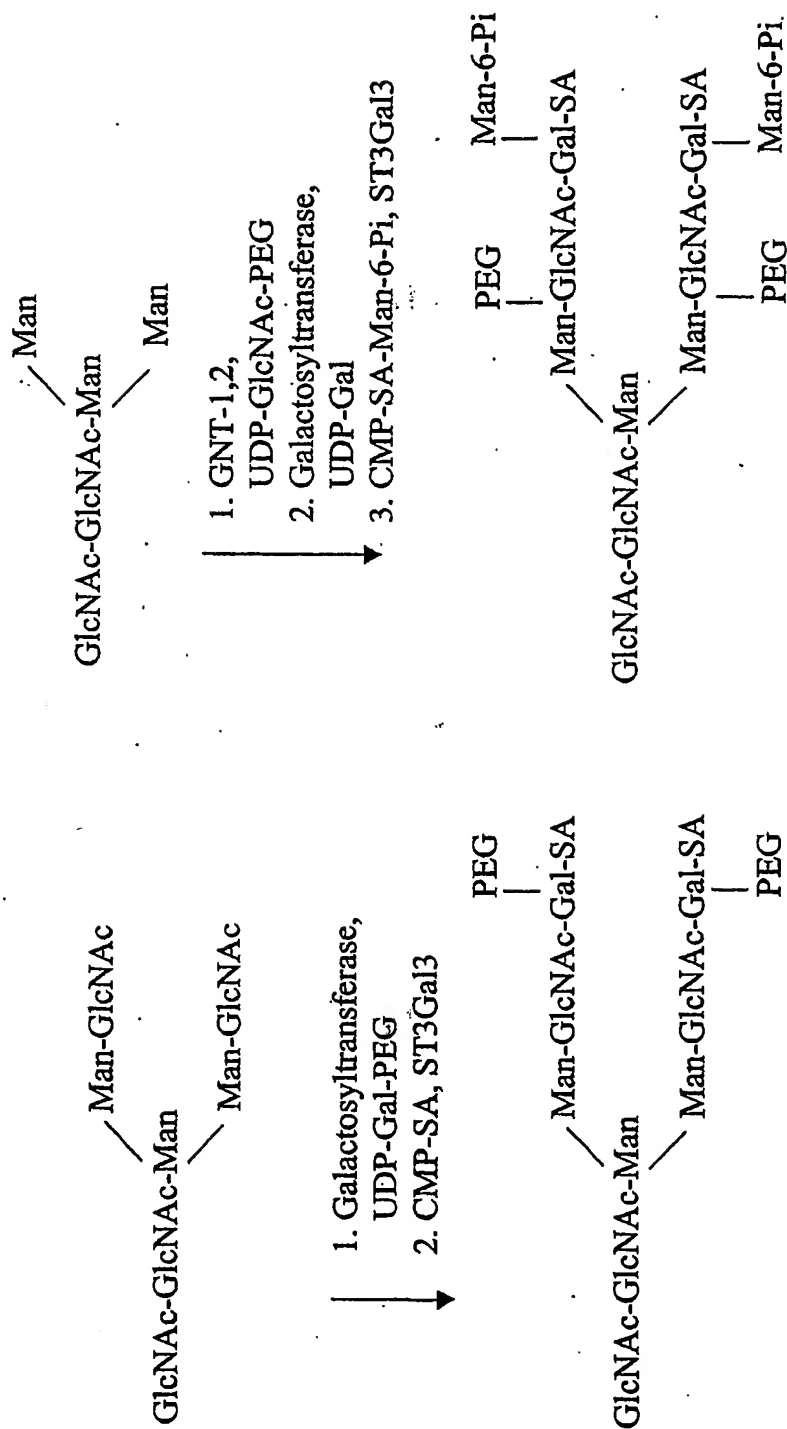


FIG. 22

25/498





26/498

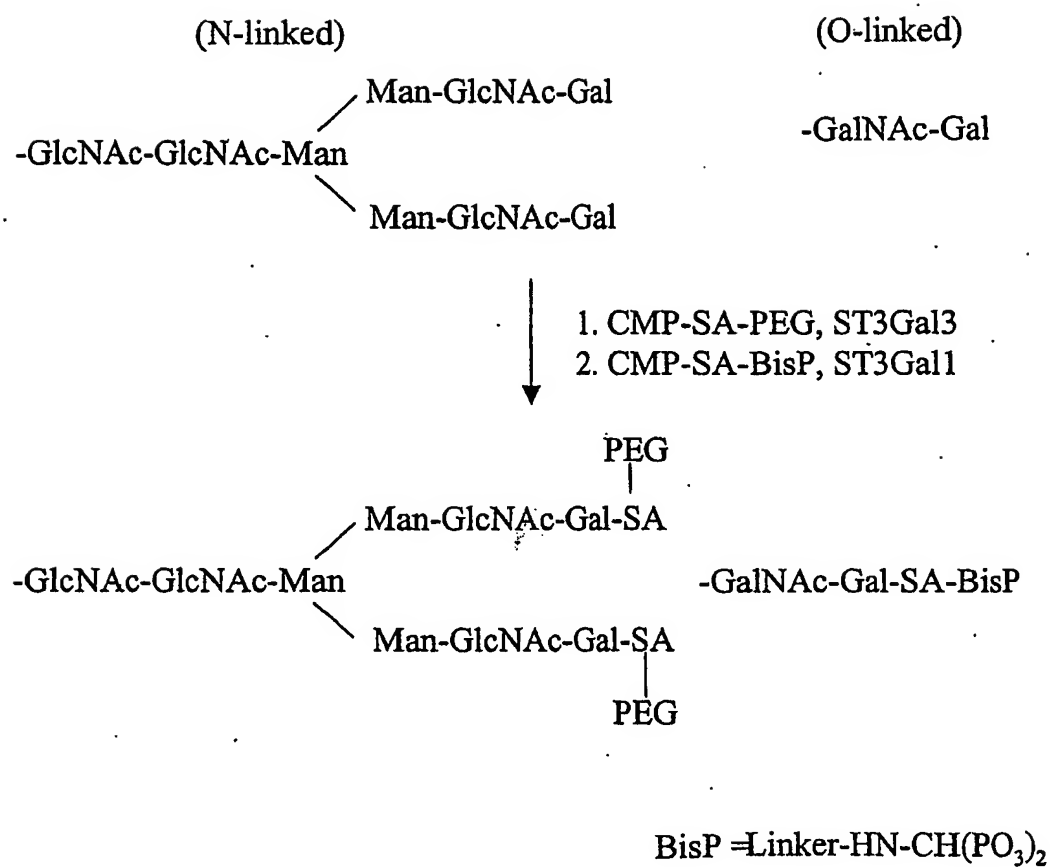


FIG. 23C

27/498

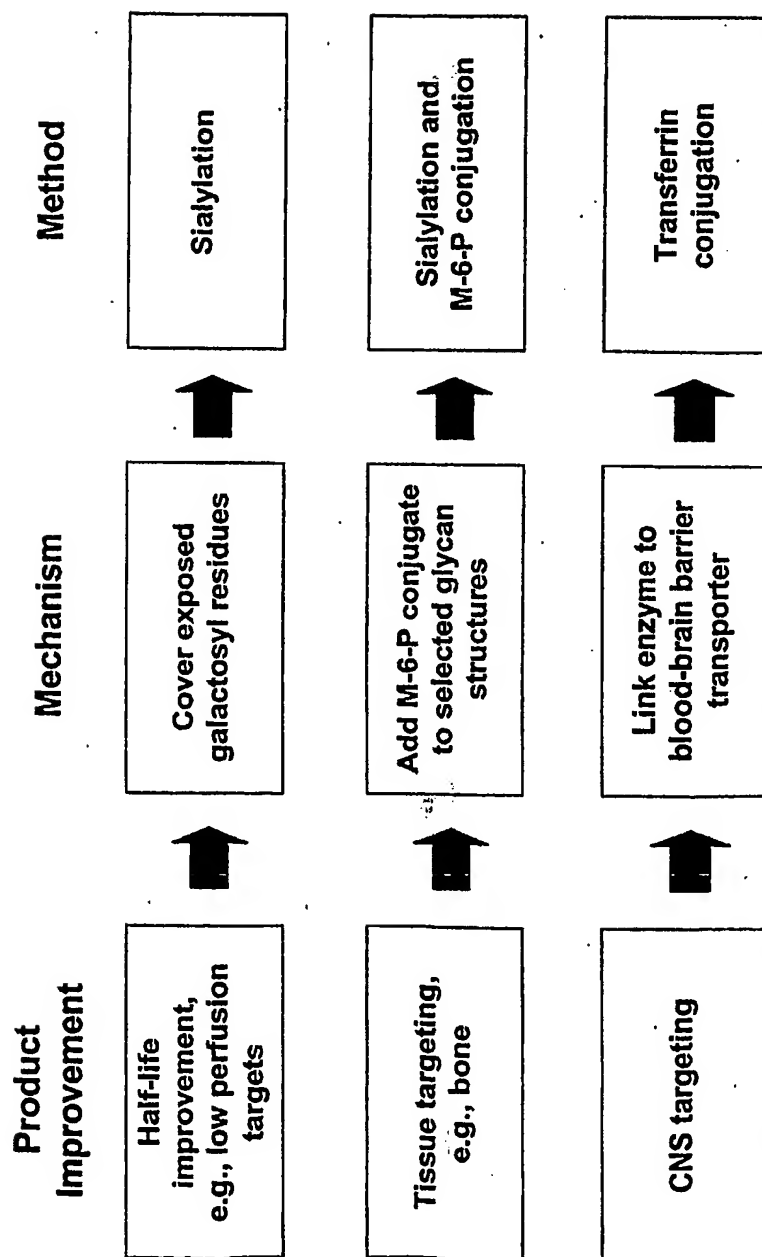


FIG. 24

28/498

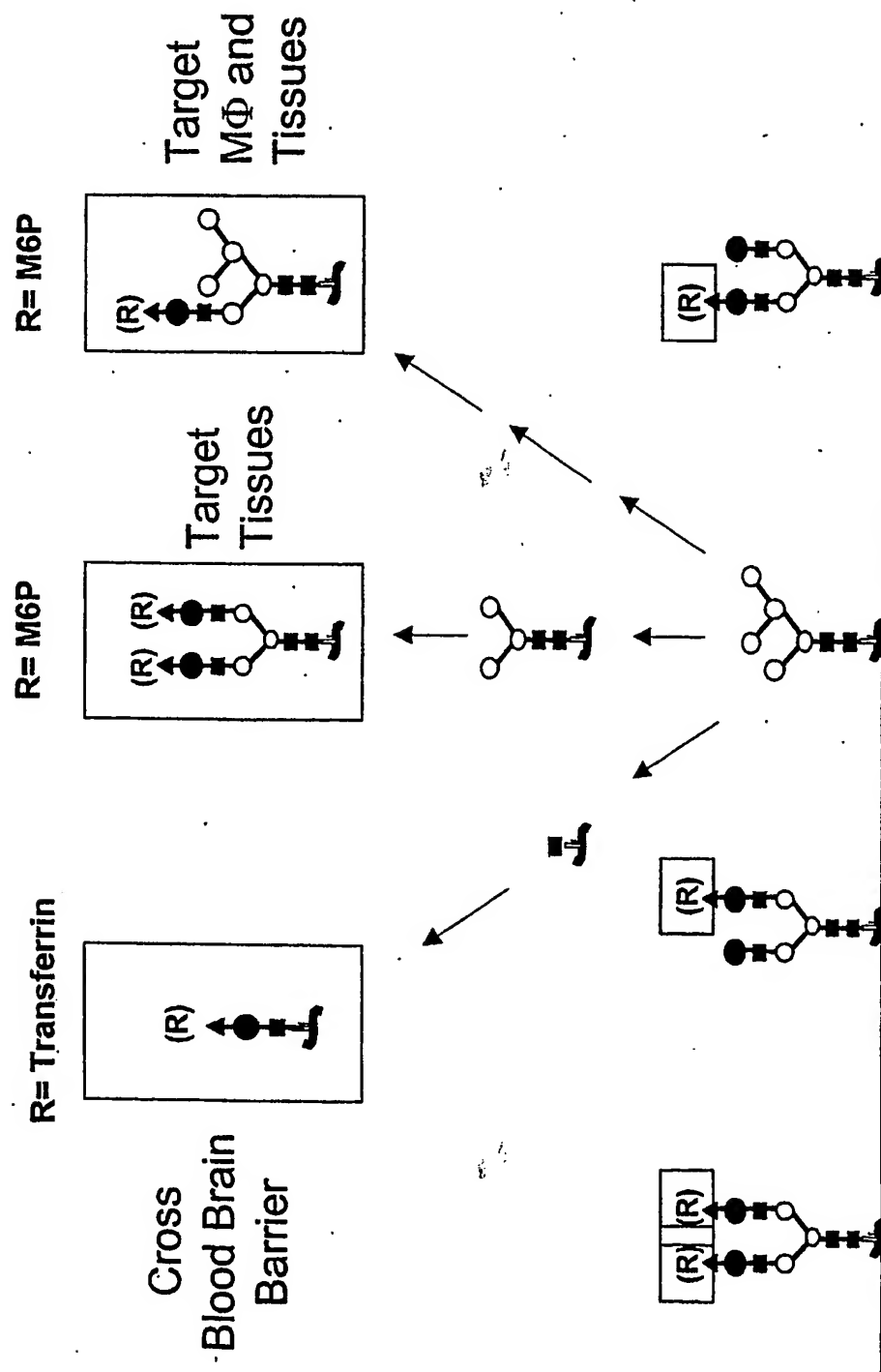


FIG. 25

29/498

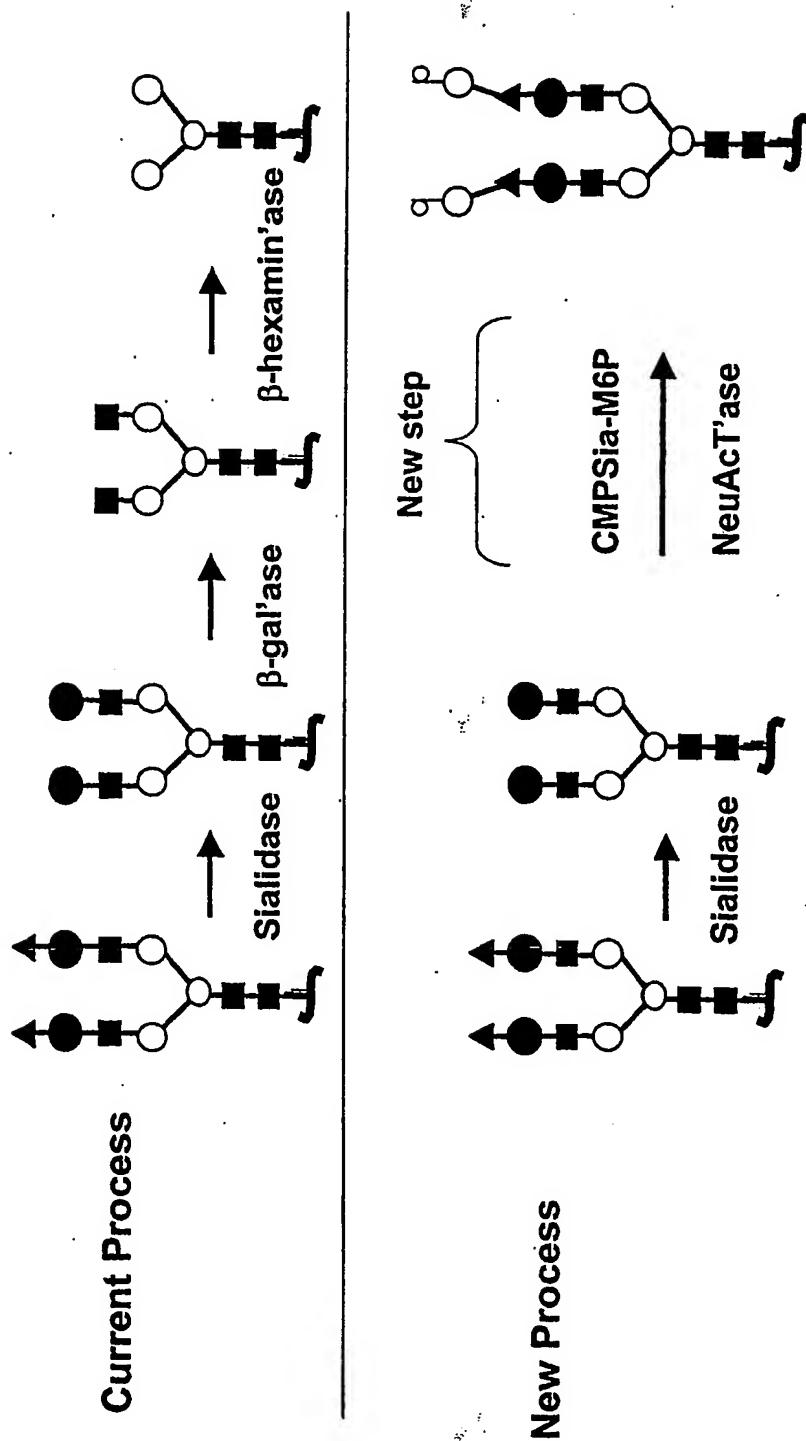


FIG. 26

30/498

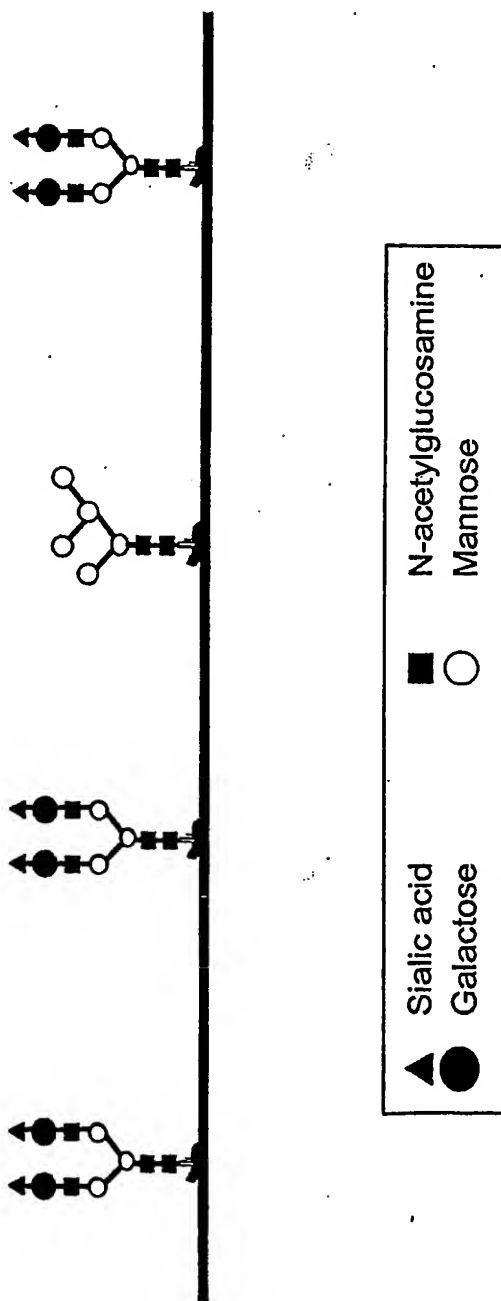


FIG. 27

## 31/498

12AP1/E5 -- Viventia Biotech	AI-201 -- AutoImmune
1964 -- Aventis	AI-301 -- AutoImmune
20K growth hormone -- AMUR	AIDS vaccine -- ANRS, CIBG, Hersed
28P6/E6 -- Viventia Biotech	Biomed, Hollis-Eden, Rome, United
3-Hydroxyphthaloyl-beta-lactoglobulin --	Biomedical, American Home Products,
4-IBB ligand gene therapy --	Maxygen
64-Cu MAb conjugate TETA-1A3 --	airway receptor ligand -- IC Innovations
Mallinckrodt Institute of Radiology	AJvW 2 -- Ajinomoto
64-Cu MAb conjugate TETA-cT84.66	AK 30 NGF -- Alkermes
64-Cu Trastuzumab TETA conjugate --	Albuferon -- Human Genome Sciences
Genentech	albumin -- Biogen, DSM Anti-Infectives,
A 200 -- Amgen	Genzyme Transgenics, PPL Therapeutics,
A10255 -- Eli Lilly	TranXenoGen, Welfide Corp.
A1PDX -- Hedral Therapeutics	aldesleukin -- Chiron
A6 -- Angstrom	alefacept -- Biogen
aaAT-III -- Genzyme	Alemtuzumab
Abciximab -- Centocor	Allergy therapy -- ALK-Abello/Maxygen,
ABI.001 -- Atlantic BioPharmaceuticals	ALK-Abello/RP Scherer
ABT-828 -- Abbott	allergy vaccines -- Allergy Therapeutics
Accutin	Alnidofibatide -- Aventis Pasteur
Actinohivin	Alnorine -- SRC VB VECTOR
activin -- Biotech Australia, Human	ALP 242 -- Gruenenthal
Therapeutics, Curis	Alpha antitrypsin -- Arriva/Hyland
AD 439 -- Tanox	Immuno/ProMetic/Protease Sciences
AD 519 -- Tanox	Alpha-1 antitrypsin -- Cutter, Bayer, PPL
Adalimumab -- Cambridge Antibody Tech.	Therapeutics, Profile, ZymoGenetics,
Adenocarcinoma vaccine -- Biomira -- NIS	Arriva
Adenosine deaminase -- Enzond	Alpha-1 protease inhibitor -- Genzyme
Adenosine A2B receptor antagonists --	Transgenics, Welfide Corp.
Adenosine Therapeutics	Alpha-galactose fusion protein --
ADP-001 -- Axis Genetics	Immunomedics
AF 13948 -- Affymax	Alpha-galactosidase A -- Research
Afelimomab -- Knoll	Corporation Technologies, Genzyme
AFP-SCAN -- Immunomedics	Alpha-glucosidase -- Genzyme, Novazyme
AG 2195 -- Corixa	Alpha-lactalbumin
agalsidase alfa -- Transkaryotic Therapies	Alpha-L-iduronidase -- Transkaryotic
agalsidase beta -- Genzyme	Therapies, BioMarin
AGENT-- Antisoma	alteplase -- Genentech
AI 300 -- AutoImmune	alvircept sudotox -- NIH
AI-101 -- Teva	ALX-0600, a GLP-2 agonist -- NPS Allelix
AI-102 -- Teva	Corp.

FIG. 28A

32/498

ALX1-11 --sNPS Pharmaceuticals	Anti-alphav $\beta$ 3 integrin MAb -- Applied
Alzheimer's disease gene therapy	Molecular Evolution
AM-133 -- AMRAD	Anti-angiogenesis monoclonal antibodies --
Amb a 1 immunostim conj. -- Dynavax	KS Biomedix/Schering AG
AMD 3100 -- AnorMED -- NIS	Anti-B4 MAb-DC1 conjugate -- ImmunoGen
AMD 3465 -- AnorMED -- NIS	Anti-B7 antibody PRIMATIZED -- IDEC
AMD 3465 -- AnorMED -- NIS	Anti-B7-1 MAb 16-10A1
AMD Fab -- Genentech	Anti-B7-1 MAb 1G10
Amediplase -- Menarini, Novartis	Anti-B7-2 MAb GL-1
AM-F9	Anti-B7-2-gelonin immunotoxin --
Amoebiasis vaccine	Antibacterials/antifungals --
Amphiregulin -- Octagene	Diversa/IntraBiotics
anakinra -- Amgen	Anti-beta-amyloid monoclonal antibodies --
analgesic -- Nobex	Cambridge Antibody Tech., Wyeth-Ayerst
ancestim -- Amgen	Anti-BLyS antibodies -- Cambridge
AnergiX.RA -- Corixa, Organon	Antibody Tech. /Human Genome Sciences
Angiocidin -- InKine	Antibody-drug conjugates -- Seattle
angiogenesis inhibitors -- ILEX	Genetics/Eos
AngioMab -- Antisoma	Anti-C5 MAb BB5-1 -- Alexion
Angiopoietins -- Regeneron/Procter &	Anti-C5 MAb N19-8 -- Alexion
Gamble	Anti-C8 MAb
angiostatin -- EntreMed	anticancer cytokines -- BioPulse
Angiostatin/endostatin gene therapy --	anticancer matrix -- Telios Integra
Genetix Pharmaceuticals	Anticancer monoclonal antibodies -- ARIUS,
angiotensin-II, topical -- Maret	Immunex
Anthrax -- EluSys Therapeutics/US Army	anticancer peptides -- Maxygen, Micrologix
Medical Research Institute	Anticancer prodrug Tech. -- Alexion
Anthrax vaccine	Antibody Technologies
Anti platelet-derived growth factor D human	anticancer Troy-Bodies -- Affite -- Affitech
monoclonal antibodies -- CuraGen	anticancer vaccine -- NIH
Anti-17-1A MAb 3622W94 --	anticancers -- Epimmune
GlaxoSmithKline	Anti-CCR5/CXCR4 sheep MAb -- KS
Anti-2C4 MAb -- Genentech	Biomedix Holdings
anti-4-1BB monoclonal antibodies -- Bristol-	Anti-CD11a MAb KBA --
Myers Squibb	Anti-CD11a MAb M17
Anti-Adhesion Platform Tech. -- Cytovax	Anti-CD11a MAb TA-3 --
Anti-adipocyte MAb -- Cambridge Antibody	Anti-CD11a MAb WT.1 --
Tech./ObeSys	Anti-CD11b MAb -- Pharmacia
antiallergics -- Maxygen	Anti-CD11b MAb LM2
antiallergy vaccine -- Acambis	Anti-CD154 MAb -- Biogen
Anti-alpha-4-integrin MAb	Anti-CD16-anti-CD30 MAb -- Biotest

FIG. 28B

33/498

Anti-CD18 MAb -- Pharmacia	Anti-CD4 MAb -- Centocor, IDEC
Anti-CD19 MAb B43 --	Pharmaceuticals, Xenova Group
Anti-CD19 MAb -liposomal sodium butyrate	Anti-CD4 MAb 16H5
conjugate --	Anti-CD4 MAb 4162W94 -- GlaxoSmithKline
Anti-CD147	Anti-CD4 MAb B-F5 -- Diaclone
Anti-CD19 MAb-saporin conjugate --	Anti-CD4 MAb GK1-5
Anti-CD19-dsFv-PE38-immunotoxin --	Anti-CD4 MAb KT6
Anti-CD2 MAb 12-15 --	Anti-CD4 MAb OX38
Anti-CD2 MAb B-E2 -- Diaclone	Anti-CD4 MAb PAP conjugate -- Bristol-
Anti-CD2 MAb OX34 --	Myers Squibb
Anti-CD2 MAb OX54 --	Anti-CD4 MAb RIB 5-2
Anti-CD2 MAb OX55 --	Anti-CD4 MAb W3/25
Anti-CD2 MAb RM2-1	Anti-CD4 MAb YTA 3.1.2
Anti-CD2 MAb RM2-2	Anti-CD4 MAb YTS 177-9
Anti-CD2 MAb RM2-4	Anti-CD40 ligand MAb 5c8 -- Biogen
Anti-CD20 MAb BCA B20	Anti-CD40 MAb
Anti-CD20-anti-Fc alpha RI bispecific MAb --	Anti-CD40 MAb 5D12 -- Tanox
Medarex, Tenovus	Anti-CD44 MAb A3D8
Anti-CD22 MAb-saporin-6 complex --	Anti-CD44 MAb GKWA3
Anti-CD3 immunotoxin --	Anti-CD44 MAb IM7
Anti-CD3 MAb 145-2C11 -- Pharming	Anti-CD44 MAb KM81
Anti-CD3 MAb CD4IgG conjugate --	Anti-CD44 variant monoclonal antibodies --
Genentech	Corixa/Hebrew University
Anti-CD3 MAb humanised -- Protein Design,	Anti-CD45 MAb BC8-I-131
RW Johnson	Anti-CD45RB MAb
Anti-CD3 MAb WT32	Anti-CD48 MAb HuLy-m3
Anti-CD3 MAb-ricin-chain-A conjugate --	Anti-CD48 MAb WM-63
Anti-CD3 MAb-xanthine-oxidase conjugate	Anti-CD5 MAb -- Becton Dickinson
--	Anti-CD5 MAb OX19
Anti-CD30 MAb BerH2 -- Medac	Anti-CD6 MAb
Anti-CD30 MAb-saporin conjugate	Anti-CD7 MAb-PAP conjugate
Anti-CD30-scFv-ETA'-immunotoxin	Anti-CD7 MAb-ricin-chain-A conjugate
Anti-CD38 MAb AT13/5	Anti-CD8 MAb -- Amerimmune, Cytodyn,
Anti-CD38 MAb-saporin conjugate	Becton Dickinson
Anti-CD3-anti-CD19 bispecific MAb	Anti-CD8 MAb 2-43
Anti-CD3-anti-EGFR MAb	Anti-CD8 MAb OX8
Anti-CD3-anti-interleukin-2-receptor MAb	Anti-CD80 MAb P16C10 -- IDEC
Anti-CD3-anti-MOV18 MAb -- Centocor	Anti-CD80 MAb P7C10 -- ID Vaccine
Anti-CD3-anti-SCLC bispecific MAb	Anti-CD8-idarubicin conjugate
Anti-CD4 idiotype vaccine	Anti-CEA MAb CE-25
	Anti-CEA MAb MN 14 -- Immunomedics

FIG. 28C



34/498

Anti-CEA MAb MN14-PE40 conjugate -- Immunomedics	Anti-heparanase human monoclonal antibodies -- Oxford
Anti-CEA MAb T84.66-interleukin-2 conjugate	Glycosciences/Medarex
Anti-CEA sheep MAb -- KS Biomedix Holdings	Anti-hepatitis C virus human monoclonal antibodies -- XTL Biopharmaceuticals
Anti-cell surface monoclonal antibodies -- Cambridge Antibody Tech. /Pharmacia	Anti-HER-2 antibody gene therapy
Anti-c-erbB2-anti-CD3 bifunctional MAb -- Otsuka	Anti-herpes antibody -- Epicyte
Anti-CMV MAb -- Scotgen	Anti-HIV antibody -- Epicyte
Anti-complement	anti-HIV catalytic antibody -- Hesed Biomed
Anti-CTLA-4 MAb	anti-HIV fusion protein -- Idun
Anti-EGFR catalytic antibody -- Hesed Biomed	anti-HIV proteins -- Cangene
anti-EGFR immunotoxin -- IVAX	Anti-HM1-24 MAb -- Chugai
Anti-EGFR MAb -- Abgenix	Anti-hR3 MAb
Anti-EGFR MAb 528	Anti-Human-Carcinoma-Antigen MAb -- Epicyte
Anti-EGFR MAb KSB 107 -- KS Biomedix	Anti-ICAM-1 MAb -- Boehringer Ingelheim
Anti-EGFR MAb-DM1 conjugate -- ImmunoGen	Anti-ICAM-1 MAb 1A-29 -- Pharmacia
Anti-EGFR MAb-LA1 --	Anti-ICAM-1 MAb HA58
Anti-EGFR sheep MAb -- KS Biomedix	Anti-ICAM-1 MAb YN1/1.7.4
Anti-FAP MAb F19-I-131	Anti-ICAM-3 MAb ICM3 -- ICOS
Anti-Fas IgM MAb CH11	Anti-idiotypic breast cancer vaccine 11D10
Anti-Fas MAb Jo2	Anti-idiotypic breast cancer vaccine ACA14C5 --
Anti-Fas MAb RK-8	Anti-idiotypic cancer vaccine -- ImClone Systems/Merck KGaA ImClone, Viventia Biotech
Anti-Flt-1 monoclonal antibodies -- ImClone	Anti-idiotypic cancer vaccine 1A7 -- Titan
Anti-fungal peptides -- State University of New York	Anti-idiotypic cancer vaccine 3H1 -- Titan
antifungal tripeptides -- BTG	Anti-idiotypic cancer vaccine TriAb -- Titan
Anti-ganglioside GD2 antibody-interleukin-2 fusion protein -- Lexigen	Anti-idiotypic Chlamydia trachomatis vaccine
Anti-GM2 MAb -- Kyowa	Anti-idiotypic colorectal cancer vaccine -- Novartis
Anti-GM-CSF receptor monoclonal antibodies -- AMRAD	Anti-idiotypic colorectal cancer vaccine -- Onyvax
Anti-gp130 MAb -- Tosoh	Anti-idiotypic melanoma vaccine -- IDEC Pharmaceuticals
Anti-HCA monoclonal antibodies -- AltaRex/Epigen	Anti-idiotypic ovarian cancer vaccine ACA 125
Anti-hCG antibodies -- Abgenix/AVI BioPharma	Anti-idiotypic ovarian cancer vaccine AR54 - AltaRex

FIG. 28D

35/498

Anti-idiotypic ovarian cancer vaccine CA-125 – AltaRex, Biomira	Anti-L-selectin monoclonal antibodies -- Protein Design Labs, Abgenix, Stanford University
Anti-IgE catalytic antibody -- Hersed Biomed	Anti-MBL monoclonal antibodies -- Alexion/Brigham and Women's Hospital
Anti-IgE MAb E26 -- Genentech	Anti-MHC monoclonal antibodies
Anti-IGF-1 MAb	Anti-MIF antibody humanised – IDEC, Cytokine PharmaSciences
anti-inflammatory -- GeneMax	Anti-MRSA/VRSA sheep MAb -- KS Biomedix Holdings
anti-inflammatory peptide -- BTG	Anti-mu MAb -- Novartis
anti-integrin peptides -- Burnha	Anti-MUC-1 MAb
Anti-interferon-alpha-receptor MAb 64G12 -- Pharma Pacific Management	Anti-MUC 18
Anti-interferon-gamma MAb -- Protein Design Labs	Anti-Nogo-A MAb IN1
Anti-interferon-gamma polyclonal antibody - Advanced Biotherapy	Anti-nuclear autoantibodies -- Procyon
Anti-interleukin-10 MAb --	Anti-ovarian cancer monoclonal antibodies - Dompe
Anti-interleukin-12 MAb --	Anti-p185 monoclonal antibodies
Anti-interleukin-1-beta polyclonal antibody -- R&D Systems	Anti-p43 MAb
Anti-interleukin-2 receptor MAb 2A3	Antiparasitic vaccines
Anti-interleukin-2 receptor MAb 33B3-1 -- Immunotech	Anti-PDGF/bFGF sheep MAb -- KS Biomedix
Anti-interleukin-2 receptor MAb ART-18	Anti-properdin monoclonal antibodies -- Abgenix/Gliatech
Anti-interleukin-2 receptor MAb LO-Tact-1	Anti-PSMA (prostate specific membrane antigen)
Anti-interleukin-2 receptor MAb Mikbeta1	Anti-PSMA MAb J591 -- BZL Biologics
Anti-interleukin-2 receptor MAb NDS61	Anti-Rev MAb gene therapy --
Anti-interleukin-4 MAb 11B11	Anti-RSV antibodies -- Epicyte, Intracell
Anti-interleukin-5 MAb -- Wallace Laboratories	Anti-RSV monoclonal antibodies -- Medarex/MedImmune, Applied Molecular Evolution/MedImmune
Anti-interleukin-6 MAb -- Centocor, Diaclone, Pharmadigm	Anti-RSV MAb, inhalation -- Alkermes/MedImmune
Anti-interleukin-8 MAb -- Abgenix	Anti-RT gene therapy
Anti-interleukin-8 MAb -- Xenotech	Antisense K-ras RNA gene therapy
Anti-JL1 MAb	Anti-SF-25 MAb
Anti-Klebsiella sheep MAb -- KS Biomedix Holdings	Anti-sperm antibody -- Epicyte
Anti-Laminin receptor MAb-liposomal doxorubicin conjugate	Anti-Tac(Fv)-PE38 conjugate
Anti-LCG MAb -- Cytoclonal	Anti-TAPA/CD81 MAb AMP1
Anti-lipopolysaccharide MAb -- VitaResc	Anti-tat gene therapy

FIG. 28E

## 36/498

Anti-TCR-alphabeta MAb H57-597	AOP-RANTES -- Senetek
Anti-TCR-alphabeta MAb R73	Apan-CH -- Praecis Pharmaceuticals
Anti-tenascin MAb BC-4-I-131	APC-8024 -- Demegen
Anti-TGF-beta human monoclonal antibodies -- Cambridge Antibody Tech., Genzyme	ApoA-1 -- Milano, Pharmacia
Anti-TGF-beta MAb 2G7 -- Genentech	Apogen -- Alexion
Antithrombin III -- Genzyme Transgenics, Aventis, Bayer, Behringwerke, CSL, Myriad	apolipoprotein A1 -- Avanir
Anti-Thy1 MAb	Apolipoprotein E -- Bio-Tech. General
Anti-Thy1.1 MAb	Applaggin -- Biogen
Anti-tissue factor/factor VIIA sheep MAb -- KS Biomedix	aprotinin -- ProdiGene
Anti-TNF monoclonal antibodies -- Centocor, Chiron, Peptech, Pharacia, Serono	APT-070C -- AdProTech
Anti-TNF sheep MAb -- KS Biomedix Holdings	AR 177 -- Aronex Pharmaceuticals
Anti-TNFalpha MAb -- Genzyme	AR 209 -- Aronex Pharmaceuticals, Antigenics
Anti-TNFalpha MAb B-C7 -- Diaclone	AR545C
Anti-tooth decay MAb -- Planet BioTech.	ARGENT gene delivery systems -- ARIAD
Anti-TRAIL receptor-1 MAb -- Takeda	Arresten
Antitumour RNases -- NIH	ART-123 -- Asahi Kasei
Anti-VCAM MAb 2A2 -- Alexion	arylsulfatase B -- BioMarin
Anti-VCAM MAb 3F4 -- Alexion	Arylsulfatase B, Recombinant human -- BioMarin
Anti-VCAM-1 MAb	AS 1051 -- Ajinomoto
Anti-VEC MAb -- ImClone	ASI-BCL -- Intracell
Anti-VEGF MAb -- Genentech	Asparaginase - Merck
Anti-VEGF MAb 2C3	ATL-101 -- Alizyme
Anti-VEGF sheep MAb -- KS Biomedix Holdings	Atrial natriuretic peptide -- Pharis
Anti-VLA-4 MAb HP1/2 -- Biogen	Aurintricarboxylic acid-high molecular weight
Anti-VLA-4 MAb PS/2	Autoimmune disorders -- GPC
Anti-VLA-4 MAb R1-2	Biotech/MorphoSys
Anti-VLA-4 MAb TA-2	Autoimmune disorders and transplant rejection -- Bristol-Myers Squibb/Genzyme
Anti-VAP-1 human MAb	Tra
Anti-VRE sheep MAb -- KS Biomedix Holdings	Autoimmune disorders/cancer -- Abgenix/Chiron, CuraGen
ANUP -- TranXenoGen	Autotaxin
ANUP-1 -- Pharis	Avicidin -- NeoRx
	axogenesis factor-1 -- Boston Life Sciences
	Axokine -- Regeneron
	B cell lymphoma vaccine -- Biomira
	B7-1 gene therapy --
	BABS proteins -- Chiron

FIG. 28F

37/498

BAM-002 -- Novelos Therapeutics	BMP 2 -- Genetics Institute/Medtronic-
Basiliximab (anti CD25 MAb) -- Novartis	Sofamor Danek, Genetics Institute/
Bay-16-9996 -- Bayer	Collagenesis, Genetics
Bay-39-9437 -- Bayer	Institute/Yamanouch
Bay-50-4798 -- Bayer	BMP 2 gene therapy
BB-10153 -- British Biotech	BMP 52 -- Aventis Pasteur, Biopharm
BBT-001 -- Bolder BioTech.	BMP-2 -- Genetics Institute
BBT-002 -- Bolder BioTech.	BMS 182248 -- Bristol-Myers Squibb
BBT-003 -- Bolder BioTech.	BMS 202448 -- Bristol-Myers Squibb
BBT-004 -- Bolder BioTech.	bone growth factors -- IsoTis
BBT-005 -- Bolder BioTech.	BPC-15 -- Pfizer
BBT-006 -- Bolder BioTech.	brain natriuretic peptide --
BBT-007 -- Bolder BioTech.	Breast cancer -- Oxford
BCH-2763 -- Shire	GlycoSciences/Medarex
BCSF -- Millenium Biologix	Breast cancer vaccine -- Therion Biologics,
BDNF -- Regeneron -- Amgen	Oregon
Becaplermin -- Johnson & Johnson, Chiron	BSSL -- PPL Therapeutics
Bectumomab -- Immunomedics	BST-2001 -- BioStratum
Beriplast -- Aventis	BST-3002 -- BioStratum
Beta-adrenergic receptor gene therapy --	BTI 322 --
University of Arkansas	butyrylcholinesterase -- Shire
bFGF -- Scios	C 6822 -- COR Therapeutics
BI 51013 -- Behringwerke AG	C1 esterase inhibitor -- Pharming
BIBH 1 -- Boehringer Ingelheim	C3d adjuvant -- AdProTech
BIM-23190 -- Beaufour-Ipsen	CAB-2.1 -- Millennium
birch pollen immunotherapy -- Pharmacia	calcitonin -- Inhale Therapeutics Systems,
bispecific fusion proteins -- NIH	Aventis, Genetronics, TranXenoGen,
Bispecific MAb 2B1 -- Chiron	Unigene, Rhone Poulenc Rohrer
Bitistatin	calcitonin -- oral -- Nobex, Emisphere,
BIWA 4 -- Boehringer Ingelheim	Pharmaceutical Discovery
blood substitute -- Northfield, Baxter Intl.	Calcitonin gene-related peptide -- Asahi
BLP-25 -- Biomira	Kasei -- Unigene
BLS-0597 -- Boston Life Sciences	calcitonin, human -- Suntory
BLyS -- Human Genome Sciences	calcitonin, nasal -- Novartis, Unigene
BLyS radiolabelled -- Human Genome	calcitonin, Panoderm -- Elan
Sciences	calcitonin, Peptitrol -- Shire
BM 06021 -- Boehringer Mannheim	calcitonin, salmon -- Therapicon
BM-202 -- BioMarin	calin -- Biopharm
BM-301 -- BioMarin	Calphobindin I
BM-301 -- BioMarin	calphobindin I -- Kowa
BM-302 -- BioMarin	calreticulin -- NYU

FIG. 28G

38/498

Campath-1G	CD4 fusion toxin -- Senetek
Campath-1M	CD4 IgG -- Genentech
cancer therapy -- Cangene	CD4 receptor antagonists --
cancer vaccine -- Aixlie, Aventis Pasteur,	Pharmacopeia/Progenics
Center of Molecular Immunology, YM	CD4 soluble -- Progenics
BioSciences, Cytos, Genzyme,	CD4, soluble -- Genzyme Transgenics
Transgenics, GlobelImmune, Igeneon,	CD40 ligand -- Immunex
ImClone, Virogenetics, InterCell, Iomai,	CD4-ricin chain A -- Genentech
Jenner Biotherapies, Memorial Sloan-	CD59 gene therapy -- Alexion
Kettering Cancer Center, Sydney Kimmel	CD8 TIL cell therapy -- Aventis Pasteur
Cancer Center, Novavax, Protein	CD8, soluble -- Avidex
Sciences, Argonex, SIGA	CD95 ligand -- Roche
Cancer vaccine ALVAC-CEA B7.1 --	CDP 571 -- Celltech
Aventis Pasteur/Therion Biologics	CDP 850 -- Celltech
Cancer vaccine CEA-TRICOM -- Aventis	CDP-860 (PEG-PDGF MAb) -- Celltech
Pasteur/Therion Biologics	CDP 870 -- Celltech
Cancer vaccine gene therapy -- Cantab	CDS-1 -- Ernest Orlando
Pharmaceuticals	Cedelizumab -- Ortho-McNeil
Cancer vaccine HER-2/neu -- Corixa	Cetermin -- Insmad
Cancer vaccine THERATOPE -- Biomira	CETP vaccine -- Avant
cancer vaccine, PolyMASC -- Valentis	Cetorelix
Candida vaccine -- Corixa, Inhibitex	Cetuximab
Canstatin -- ILEX	CGH 400 -- Novartis
CAP-18 -- Panorama	CGP 42934 -- Novartis
Cardiovascular gene therapy -- Collateral	CGP 51901 -- Tanox
Therapeutics	CGRP -- Unigene
carperitide -- Suntory	CGS 27913 -- Novartis
Casocidin-1 -- Pharis	CGS 32359 -- Novartis
CAT 152 -- Cambridge Antibody Tech.	Chagas disease vaccine -- Corixa
CAT 192 -- Cambridge Antibody Tech.	chemokines -- Immune Response
CAT 213 -- Cambridge Antibody Tech.	CHH 380 -- Novartis
Catalase -- Enzon	chitinase -- Genzyme, ICOS
Cat-PAD -- Circassia	Chlamydia pneumoniae vaccine -- Antex
CB 0006 -- Celltech	Biologics
CCK(27-32) -- Akzo Nobel	Chlamydia trachomatis vaccine -- Antex
CCR2-641 -- NIH	Biologics
CD, Procept -- Paligent	Chlamydia vaccine -- GlaxoSmithKline
CD154 gene therapy	Cholera vaccine CVD 103-HgR -- Swiss
CD39 -- Immunex	Serum and Vaccine Institute Berne
CD39-L2 -- Hyseq	Cholera vaccine CVD 112 -- Swiss Serum
CD39-L4 -- Hyseq	and Vaccine Institute Berne

FIG. 28H

39/498

Cholera vaccine inactivated oral -- SBL Vaccin	CRL 1605 -- CytRx
Chrysalin -- Chrysalis BioTech.	CS-560 -- Sankyo
CI-782 -- Hitachi Kase	CSF -- ZymoGenetics
Ciliary neurotrophic factor -- Fidia, Roche	CSF-G -- Hangzhou, Dong-A, Hanmi
CIM project -- Active Biotech	CSF-GM -- Cangene, Hunan, LG Chem
CL 329753 -- Wyeth-Ayerst	CSF-M -- Zarix
CL22, Cobra -- ML Laboratories	CT 1579 -- Merck Frosst
Clenoliximab -- IDEC	CT 1786 -- Merck Frosst
Clostridium difficile antibodies -- Epicyte	CT-112 <sup>A</sup> -- BTG
clotting factors -- Octagene	CTB-134L -- Xenova
CMB 401 -- Celltech	CTC-111 -- Kaketsuken
CNTF -- Sigma-Tau	CTGF -- FibroGen
Cocaine abuse vaccine -- Cantab, ImmuLogic, Scripps	CTLA4-Ig -- Bristol-Myers Squibb
coccidiomycosis vaccine -- Arizo	CTLA4-Ig gene therapy --
collagen -- Type I -- Pharming	CTP-37 -- AVI BioPharma
Collagen formation inhibitors -- FibroGen	C-type natriuretic peptide -- Suntory
Collagen/hydroxyapatite/bone growth factor -- Aventis Pasteur, Biopharm, Orquest	CVS 995 -- Corvas Intl.
collagenase -- BioSpecifics	CX 397 -- Nikko Kyodo
Colorectal cancer vaccine -- Wistar Institute	CY 1747 -- Epimmune
Component B, Recombinant -- Serono	CY 1748 -- Epimmune
Connective tissue growth factor inhibitors -- FibroGen/Taisho	Cyanovirin-N
Contortrostatin	Cystic fibrosis therapy -- CBR/IVAX
contraceptive vaccine -- Zonagen	CYT 351
Contraceptive vaccine hCG	cytokine Traps -- Regeneron
Contraceptive vaccine male reversible -- IMMUCON	cytokines -- Enzon, Cytoclonal
Contraceptive vaccine zona pellucida -- Zonagen	Cytomegalovirus glycoprotein vaccine -- Chiron, Aquila Biopharmaceuticals, Aventis Pasteur, Virogenetics
Copper-64 labelled MAb TETA-1A3 -- NCI	Cytomegalovirus vaccine live -- Aventis Pasteur
Coralyne	Cytosine deaminase gene therapy -- GlaxoSmithKline
Corsevin M	DA-3003 -- Dong-A
C-peptide analogues -- Schwarz	DAB389interleukin-6 -- Senetek
CPI-1500 -- Consensus	DAB389interleukin-7
CRF -- Neurobiological Tech.	DAC:GLP-2 -- ConjuChem, Inc.
cRGDfV pentapeptide --	Daclizumab (anti-IL2R MAb) -- Protein Design Labs
CRL 1095 -- CytRx	DAMP <sup>A</sup> -- Incyte Genomics
CRL 1336 -- CytRx	Daniplestim -- Pharmacia
	darbepoetin alfa -- Amgen

FIG. 28I

40/498

DBI-3019 -- Diabetogen	dural graft matrix -- Integra
DCC -- Genzyme	Duteplase -- Baxter Intl.
DDF -- Hyseq	DWP-401 -- Daewoong
decorin -- Integra, Telios	DWP-404 -- Daewoong
defensins -- Large Scale Biology	DWP-408 -- Daewoong
DEGR-VIIa	Dx 88 (Epi-KAL2) -- Dyax
DeImmunised antibody 3B6/22 AGEN	Dx 890 (elastin inhibitors) -- Dyax
Deimmunised anti-cancer antibodies -- Biovation/Viragen	E coli O157 vaccine -- NIH
Dendroamide A	E21-R -- BresaGen
Dengue vaccine -- Bavarian Nordic, Merck	Eastern equine encephalitis virus vaccine -- Echicetin --
denileukin diftitox -- Ligand	Echinhibin 1 --
DES-1101 -- Desmos	Echistatin -- Merck
desirudin -- Novartis	Echitamine --
desmopressin -- Unigene	Ecromeximab -- Kyowa Hakko
Desmoteplase -- Merck, Schering AG	EC-SOD -- PPL Therapeutics
Destabilase	Eculizumab (5G1.1) -- Alexion
Diabetes gene therapy -- DeveloGen, Pfizer	EDF -- Ajinomoto
Diabetes therapy -- Crucell	EDN derivative -- NIH
Diabetes type 1 vaccine -- Diamyd Therapeutics	EDNA -- NIH
DiaCIM -- YM BioSciences	Edobacomab -- XOMA
dialytic oligopeptides -- Research Corp	Edrecolomab -- Centocor
Diamyd -- Diamyd Therapeutics	EF 5077
DiaPep227-- Pepgen	Efalizumab -- Genentech
DiavaX -- Corixa	EGF fusion toxin -- Seragen, Ligand
Digoxin MAb -- Glaxo	EGF-P64k vaccine -- Center of Molecular Immunology
Diphtheria tetanus pertussis-hepatitis B vaccine -- GlaxoSmithKline	EL 246 -- LigoCyte
DIR therapy -- Solis Therapeutics --	elastase inhibitor -- Synergen
DNase -- Genentech	elcatonin -- Therapicon
Dornase alfa -- Genentech	EMD 72000 -- Merck KGaA
Dornase alfa, inhalation -- Genentech	Emdogain -- BIORA
Doxorubicin-anti-CEA MAb conjugate -- Immunomedics	emfilermin -- AMRAD
DP-107 -- Trimeris	Emoctakin -- Novartis
drotrecogin alfa -- Eli Lilly	enamel matrix protein -- BIORA
DTctGMCSF	Endo III -- NYU
DTP-polio vaccine -- Aventis Pasteur	endostatin -- EntreMed, Pharis
DU 257-KM231 antibody conjugate -- Kyowa	Enhancins -- Micrologix
	Enlimomab -- Isis Pharm.
	Enoxaparin sodium -- Pharmuka

FIG. 28J

41/498

enzyme linked antibody nutrient depletion therapy -- KS Biomedix Holdings	Factor IX gene therapy -- Cell Genesys
Eosinophil-derived neutralizing agent --	Factor VII -- Novo Nordisk, Bayer, Baxter Intl.
EP-51216 -- Asta Medica	Factor VIIa -- PPL Therapeutics, ZymoGenetics
EP-51389 -- Asta Medica	Factor VIII -- Bayer Genentech, Beaufour-Ipsen, CLB, Inex, Octagen, Pharmacia, Pharming
EPH family ligands -- Regeneron	Factor VIII -- PEGylated -- Bayer
Epidermal growth factor -- Hitachi Kasei, Johnson & Johnson	Factor VIII fragments -- Pharmacia
Epidermal growth factor fusion toxin -- Senetek	Factor VIII gene therapy -- Targeted Genetics
Epidermal growth factor-genistein --	Factor VIII sucrose formulation -- Bayer, Genentech
EPI-HNE-4 -- Dyax	Factor VIII-2 -- Bayer
EPI-KAL2 -- Dyax	Factor VIII-3 -- Bayer
Epoetin-alfa -- Amgen, Dragon Pharmaceuticals, Nanjing Huaxin	Factor Xa inhibitors -- Merck, Novo Nordisk, Mochida
Epratuzumab -- Immunomedics	Factor XIII -- ZymoGenetics
Epstein-Barr virus vaccine -- Aviron/SmithKline Beecham, Bioreserach	Factors VIII and IX gene therapy -- Genetics Institute/Targeted Genetics
Eptacog alfa -- Novo Nordisk	Famoxin -- Genset
Eptifibatide -- COR Therapeutics	Fas (delta) TM protein -- LXR BioTech.
erb-38 --	Fas TR -- Human Genome Sciences
Erlizumab -- Genentech	Felvizumab -- Scotgen
erythropoietin -- Alkermes, ProLease, Dong-A, Elanex, Genetics Institute, LG Chem, Protein Sciences, Serono, Snow Brand, SRC VB VECTOR, Transkaryotic Therapies	FFR-VIIa -- Novo Nordisk
Erythropoietin Beta -- Hoffman La Roche	FG-001 -- F-Gene
Erythropoietin/Epoetin alfa -- Chugai	FG-002 -- F-Gene
Escherichia coli vaccine -- North American Vaccine, SBL Vaccin, Swiss Serum and Vaccine Institute Berne	FG-004 -- F-Gene
etanercept -- Immunex	FG-005 -- F-Gene
examorelin -- Mediolanum	FGF + fibrin -- Repair
Exendin 4 -- Amylin	Fibrimage -- Bio-Tech. General
exonuclease VII	fibrin-binding peptides -- ISIS Innovation
F 105 -- Centocor	fibrinogen -- PPL Therapeutics, Pharming
F-992 -- Fornix	fibroblast growth factor -- Chiron, NYU, Ramot, ZymoGenetics
Factor IX -- Alpha Therapeutics, Welfide Corp., CSL, enetics Institute/AHP, Pharmacia, PPL Therapeutics	fibrolase conjugate -- Schering AG
	Filgrastim -- Amgen
	filgrastim -- PDA modified -- Xencor
	FLT-3 ligand -- Immunex
	FN18 CRM9 --

FIG. 28K



42/498

follistatin -- Biotech Australia, Human Therapeutics	Glucocerebrosidase -- Genzyme
follitropin alfa -- Alkermes, ProLease, PowderJect, Serono, Akzo Nobel	glutamate decarboxylase -- Genzyme Transgenics
Follitropin Beta -- Bayer, Organon	Glycoprotein S3 -- Kureha
FP 59	GM-CSF -- Immuhex
FSH -- Ferring	GM-CSF tumour vaccine -- PowderJect
FSH + LH -- Ferring	GnRH immunotherapeutic -- Protherics
F-spondin -- CeNeS	Goserelin (LhRH antagonist) -- AstraZeneca
fusion protein delivery system -- UAB Research Foundation	gp75 antigen -- ImClone
fusion toxins -- Boston Life Sciences	gp96 -- Antigenics
G 5598 -- Genentech	GPI 0100 -- Galenica
GA-II -- Transkaryotic Therapies	GR 4991W93 -- GlaxoSmithKline
Gamma-interferon analogues -- SRC VB VECTOR	Granulocyte colony-stimulating factor -- Dong-A
Ganirelix -- Roche	Granulocyte colony-stimulating factor conjugate
gastric lipase -- Meristem	grass allergy therapy -- Dynavax
Gavilimomab --	GRF1-44 -- ICN
G-CSF -- Amgen, SRC VB VECTOR	Growth Factor -- Chiron, Atrigel, Atrix, Innogenetics, ZymoGenetics, Novo
GDF-1 -- CeNeS	growth factor peptides -- Biotherapeutics
GDF-5 -- Biopharm	growth hormone -- LG Chem
GDNF (glial derived neurotrophic factor) -- Amgen	growth hormone, Recombinant human -- Serono
gelsolin -- Biogen	GT 4086 -- Giatech
Gemtuzumab ozogamicin -- Celltech	GW 353430 -- GlaxoSmithKline
Gene-activated epoetin-alfa -- Aventis Pharma -- Transkaryotic Therapies	GW-278884 -- GlaxoSmithKline
Glanzmann thrombasthenia gene therapy --	H 11 -- Viventia Biotech
Glatiramer acetate -- Yeda	H5N1 influenza A virus vaccine -- Protein Sciences
glial growth factor 2 -- CeNeS	haemoglobin -- Biopure
GLP-1 -- Amylin, Suntory, TheraTech, Watson	haemoglobin 3011, Recombinant -- Baxter Healthcare
GLP-1 peptide analogues -- Zealand Pharmaceuticals	haemoglobin crosfumaril -- Baxter Intl.
GLP-2 -- Novo Nordisk, Ontario, Inc., Suntory Limited	haemoglobin stabilized -- Ajinomoto
glucagon -- Eli Lilly, ZymoGenetics	haemoglobin, recombinant -- Apex
Glucagon-like peptide-1 7-36 amide -- Suntory	HAF -- Immune Response
Glucogen-like peptide -- Amylin	Hantavirus vaccine
	HB 19
	HBNF -- Regeneron
	HCC-1 -- Pharis

FIG. 28L

43/498

hCG -- Milkhaus	Herpes simplex glycoprotein DNA vaccine --
hCG vaccine -- Zonagen	Merck, Wyeth-Lederle Vaccines-Malvern,
HE-317 -- Hollis-Eden Pharmaceuticals	Genentech, GlaxoSmithKline, Chiron,
Heat shock protein cancer and influenza	Takeda
vaccines -- StressGen	Herpes simplex vaccine -- Cantab
Helicobacter pylori vaccine -- Acambis,	Pharmaceuticals, CEL-SCI, Henderson
AstraZeneca/CSL, Chiron, Provalis	Morley
Helistat-G -- GalaGen	Herpes simplex vaccine live -- ImClone
Hemolink -- Hemosol	Systems/Wyeth-Lederle, Aventis Pasteur
hepapoietin -- Snow Brand	HGF derivatives -- Dompe
heparanase -- InSight	hIAPP vaccine -- Crucell
heparinase I -- Ibex	Hib-hepatitis B vaccine -- Aventis Pasteur
heparinase III -- Ibex	HIC 1
Hepatitis A vaccine -- American Biogenetic	HIP-- Altachem
Sciences	Hirudins -- Biopharma, Cangene, Dongkook,
Hepatitis A vaccine inactivated	Japan Energy Corporation, Pharmacia
Hepatitis A vaccine Nothav -- Chiron	Corporation, SIR International, Sanofi-
Hepatitis A-hepatitis B vaccine --	Synthelabo, Sotragene, Rhein Biotech
GlaxoSmithKline	HIV edible vaccine -- ProdiGene
hepatitis B therapy -- Tripep	HIV gp120 vaccine -- Chiron, Ajinomoto,
Hepatitis B vaccine -- Amgen, Chiron SpA,	GlaxoSmithKline, ID Vaccine, Progenics,
Meiji Milk, NIS, Prodeva, PowderJect,	VaxGen
Rhein Biotech	HIV gp120 vaccine gene therapy --
Hepatitis B vaccine recombinant -- Evans	HIV gp160 DNA vaccine -- PowderJect,
Vaccines, Eptec Combiotech, Genentech,	Aventis Pasteur, Oncogen, Hyland
MedImmune, Merck Sharp & Dohme,	Immuno, Protein Sciences
Rhein Biotech, Shantha Biotechnics,	HIV gp41 vaccine -- Panacos
Vector, Yeda	HIV HGP-30W vaccine -- CEL-SCI
Hepatitis B vaccine recombinant TGP 943 --	HIV immune globulin -- Abbott, Chiron
Takeda	HIV peptides -- American Home Products
Hepatitis C vaccine -- Bavarian Nordic,	HIV vaccine -- Applied bioTech., Axis
Chiron, Innogenetics Acambis,	Genetics, Biogen, Bristol-Myers Squibb,
Hepatitis D vaccine -- Chiron Vaccines	Genentech, Korea Green Cross, NIS,
Hepatitis E vaccine recombinant --	Oncogen, Protein Sciences Corporation,
Genelabs/GlaxoSmithKline, Novavax	Terumo, Tonen Corporation, Wyeth-
hepatocyte growth factor -- Panorama,	Ayerst, Wyeth-Lederle Vaccines-Malvern,
Sosei	Advanced BioScience Laboratories,
hepatocyte growth factor kringle fragments -	Bavarian Nordic, Bavarian Nordic/Statens
- EntreMed	Serum Institute, GeneCure, Immune
Her-2/Neu peptides -- Corixa	Response, Progenics, Therion Biologics,
	United Biomedical, Chiron

FIG. 28M

44/498

HIV vaccine vCP1433 -- Aventis Pasteur	Human monoclonal antibodies --
HIV vaccine vCP1452 -- Aventis Pasteur	Medarex/Northwest Biotherapeutics,
HIV vaccine vCP205 -- Aventis Pasteur	Medarex/Seattle Genetics
HL-9 -- American BioScience	human netrin-1 -- Exelixis
HM-9239 -- Cytran	human papillomavirus antibodies -- Epicyte
HML-103 -- Hemosol	Human papillomavirus vaccine -- Biotech
HML-104 -- Hemosol	Australia, IDEC, StressGen
HML-105 -- Hemosol	Human papillomavirus vaccine MEDI 501 --
HML-109 -- Hemosol	MedImmune/GlaxoSmithKline
HML-110 -- Hemosol	Human papillomavirus vaccine MEDI
HML-121 -- Hemosol	503/MEDI 504 --
hNLP -- Pharis	MedImmune/GlaxoSmithKline
Hookworm vaccine	Human papillomavirus vaccine TA-CIN --
host-vector vaccines -- Henogen	Cantab Pharmaceuticals
HPM 1 -- Chugai	Human papillomavirus vaccine TA-HPV --
HPV vaccine -- MediGene	Cantab Pharmaceuticals
HSA -- Meristem	Human papillomavirus vaccine TH-GW --
HSF -- StressGen	Cantab/GlaxoSmithKline
HSP carriers --Weizmann, Yeda, Peptor	human polyclonal antibodies -- Biosite/Eos
HSPPC-70 -- Antigenics	BioTech./ Medarex
HSPPC-96, pathogen-derived -- Antigenics	human type II anti factor VIII monoclonal
HSV 863 -- Novartis	antibodies -- ThromboGenics
HTLV-I DNA vaccine	humanised anti glycoprotein Ib murine
HTLV-I vaccine	monoclonal antibodies -- ThromboGenics
HTLV-II vaccine -- Access	HumaRAD -- Intracell
HU 901 -- Tanox	HuMax EGFR -- Genmab
Hu23F2G -- ICOS	HuMax-CD4 -- Medarex
HuHMFG1	HuMax-IL15 -- Genmab
HumaLYM -- Intracell	HYB 190 -- Hybridon
Human krebs statika -- Yamanouchi	HYB 676 -- Hybridon
human monoclonal antibodies --	I-125 MAb A33 -- Celltech
Abgenix/Biogen, Abgenix/ Corixa,	Ibritumomab tiuxetan -- IDEC
Abgenix/Immunex, Abgenix/Lexicon,	IBT-9401 -- Ibex
Abgenix/ Pfizer, Athersys/Medarex,	IBT-9402 -- Ibex
Biogen/MorphoSys, CAT/Searle,	IC 14 -- ICOS
Centocor/Medarex, Corixa/Kirin Brewery,	Idarubicin anti-Ly-2.1 --
Corixa/Medarex, Eos BioTech./Medarex,	IDEC 114 -- IDEC
Eos/Xenerex, Exelixis/Protein Design	IDEC 131 -- IDEC
Labs, ImmunoGen/ Raven, Medarex/	IDEC 152 -- IDEC
B.Twelve, MorphoSys/ImmunoGen, XTL	IDM 1 -- IDM
Biopharmaceuticals/Dyax,	IDPS -- Hollis-Eden Pharmaceuticals

FIG. 28N

45/498

iduronate-2-sulfatase -- Transkaryotic Therapies	insulin -- AutoImmune, Altea, Biobras, BioSante, Bio-Tech. General, Chong Kun Dang, Emisphere, Flamel, Provalis, Rhein Biotech, TranXenoGen
IGF/IBP-2-13 -- Pharis	insulin (bovine) -- Novartis
IGN-101 -- Igeneon	insulin analogue -- Eli Lilly
IK HIR02 -- Iketon	Insulin Aspart -- Novo Nordisk
IL-11 -- Genetics Institute/AHP	insulin detemir -- Novo Nordisk
IL-13-PE38 -- NeoPharm	insulin glargine -- Aventis
IL-17 receptor -- Immunex	insulin inhaled -- Inhale Therapeutics Systems, Alkermes
IL-18BP -- Yeda	insulin oral -- Inovax
IL-1Hy1 -- Hyseq	insulin, AeroDose -- AeroGen
IL-1 $\beta$ -- Celltech	insulin, AERx -- Aradigm
IL-1 $\beta$ adjuvant -- Celltech	insulin, BEODAS -- Elan
IL-2 -- Chiron	insulin, Biphasix -- Helix
IL-2 + IL-12 -- Hoffman La-Roche	insulin, buccal -- GenereX
IL-6/sIL-6R fusion -- Hadasit	insulin, I2R -- Flemington
IL-6R derivative -- Tosoh	insulin, intranasal -- Bentley
IL-7-Dap 389 fusion toxin -- Ligand	insulin, oral -- Nobex, Unigene
IL-21 -- Novo Nordisk, ZymoGenetics	insulin, Orasome -- Endorex
IM-862 -- Cytran	insulin, ProMaxx -- Epic
IMC-1C11 -- ImClone	insulin, Quadrant -- Elan
imiglucerase -- Genzyme	insulin, recombinant -- Aventis
Immune globulin intravenous (human) -- Hoffman La Roche	insulin, Spiros -- Elan
immune privilege factor -- Proneuron	insulin, Transfersome -- IDEA
Immunocal -- Immunotec	insulin, Zymo, recombinant -- Novo Nordisk
Immunogene therapy -- Briana Bio-Tech	insulinotropin -- Scios
Immunoliposomal 5-fluorodeoxyuridine-dipalmitate --	Insulysin gene therapy --
immunosuppressant vaccine -- Aixlie	integrin antagonists -- Merck
immunotoxin -- Antisoma, NIH	interferon (Alpha2) -- SRC VB VECTOR, Viragen, Dong-A, Hoffman La-Roche, Genentech
ImmuRAIT-Re-188 -- Immunomedics	interferon -- BioMedicines, Human Genome Sciences
imreg-1 -- Imreg	interferon (Alfa-n3) -- Interferon Sciences Intl.
infertility -- Johnson & Johnson, E-TRANS	interferon (Alpha), Biphasix -- Helix
Infliximab -- Centocor	
Influenza virus vaccine -- Aventis Pasteur, Protein Sciences	
inhibin -- Biotech Australia, Human Therapeutics	
Inhibitory G protein gene therapy	
INKP-2001 -- InKine	
Inolimomab -- Diaclone	

FIG. 280

46/498

interferon (Alpha)—Amgen, BioNative,	IL-2/ diphtheria toxin -- Ligand
Novartis, Genzyme Transgenics,	Interleukin-3 -- Cangene
Hayashibara, Inhale Therapeutics	Interleukin-4 -- Immunology Ventures,
Systems, Medusa, Flamel, Dong-A,	Sanofi Winthrop, Schering-Plough,
GeneTrol, Nastech, Shantha,	Immunex/ Sanofi Winthrop, Bayer, Ono
Wassermann, LG Chem, Sumitomo,	interleukin-4 + TNF-Alpha -- NIH
Aventis, Behring EGIS, Pepgen, Servier,	interleukin-4 agonist -- Bayer
Rhein Biotech,	interleukin-4 fusion toxin -- Ligand
interferon (Alpha2A)	Interleukin-4 receptor -- Immunex, Immun
interferon (Alpha2B) -- Enzon, Schering-	Interleukin-6 -- Ajinomoto, Cangene, Yeda,
Plough, Biogen, IDEA	Genetics Institute, Novartis
interferon (Alpha-N1) -- GlaxoSmithKline	interleukin-6 fusion protein
interferon (beta) -- Rentschler, GeneTrol,	interleukin-6 fusion toxin -- Ligand, Serono
Meristem, Rhein Biotech, Toray, Yeda,	interleukin-7 -- IC Innovations
Daiichi, Mochida	interleukin-7 receptor -- Immunex
interferon (Beta1A) -- Serono, Biogen	interleukin-8 antagonists -- Kyowa
interferon (beta1A), inhale -- Biogen	Hakko/Millennium/Pfizer
interferon (β1b)-- Chiron	interleukin-9 antagonists -- Genaera
interferon (tau)-- Pepgen	Interleukin-10 -- DNAX, Schering-Plough
Interferon alfacon-1 -- Amgen	Interleukin-10 gene therapy --
Interferon alpha-2a vaccine	interleukin-12 -- Genetics Institute, Hoffman
Interferon Beta 1b -- Schering/Chiron,	La-Roche
InterMune	interleukin-13 -- Sanofi
Interferon Gamma -- Boehringer Ingelheim,	interleukin-13 antagonists -- AMRAD
Sheffield, Rentschler, Hayashibara	Interleukin-13-PE38QQR
interferon receptor , Type I -- Serono	interleukin-15 -- Immunex
interferon(Gamma1B) -- Genentech	interleukin-16 -- Research Corp
Interferon-alpha-2b + ribavirin -- Biogen,	interleukin-18 -- GlaxoSmithKline
ICN	Interleukin-18 binding protein -- Serono
Interferon-alpha-2b gene therapy --	lor-P3 -- Center of Molecular Immunology
Schering-Plough	IP-10 -- NIH
Interferon-con1 gene therapy --	IPF -- Metabolex
interleukin-1 antagonists -- Dompe	IR-501 -- Immune Response
Interleukin-1 receptor antagonist -- Abbott	ISIS 9125 -- Isis Pharmaceuticals
Bioresearch, Pharmacia	ISURF No. 1554 -- Millennium
Interleukin-1 receptor type I -- Immunex	ISURF No. 1866 -- Iowa State Univer.
interleukin-1 receptor Type II -- Immunex	ITF-1697 -- Italfarmaco
Interleukin-1 trap -- Regeneron	ixC 162 -- Ixion
Interleukin-1-alpha -- Immunex/Roche	J 695 -- Cambridge Antibody Tech.,
interleukin-2 -- SRC VB VECTOR,	Genetics Inst., Knoll
Ajinomoto, Biomira, Chiron	Jagged + FGF -- Repair

FIG. 28P

47/498

JKC-362 -- Phoenix Pharmaceuticals	leptin, 2nd-generation -- Amgen
JTP-2942 -- Japan Tobacco	leridistim -- Pharmacia
Juman monoclonal antibodies -- Medarex/Raven	leuprolide, ProMaxx -- Epic
K02 -- Axys Pharmaceuticals	leuprorelin, oral -- Unigene
Keliximab -- IDEC	LeuTech -- Papatin
Keyhole limpet haemocyanin	LEX 032 -- SuperGen
KGF -- Amgen	LiDEPT -- Novartis
KM 871 -- Kyowa	Lintuzumab (anti-CD33 MAb) -- Protein Design Labs
KPI 135 -- Scios	lipase -- Altus Biologics
KPI-022 -- Scios	lipid A vaccine -- EntreMed
Kringle 5	lipid-linked anchor Tech. -- ICRT, ID Biomedical
KSB 304	liposome-CD4 Tech. -- Sheffield
KSB-201 -- KS Biomedix	Listeria monocytogenes vaccine
L 696418 -- Merck	LMB 1
L 703801 -- Merck	LMB 7
L1 -- Acorda	LMB 9 -- Battelle Memorial Institute, NIH
L-761191 -- Merck	LM-CD45 -- Cantab Pharmaceuticals
lactoferrin -- Meristem, Pharming, Agennix	lovastatin -- Merck
lactoferrin cardio -- Pharming	LSA-3
LAG-3 -- Serono	LT- $\beta$ receptor -- Biogen
LAIT -- GEMMA	lung cancer vaccine -- Corixa
LAK cell cytotoxin -- Arizona	lusupultide -- Scios
lamellarins -- PharmaMar/University of Malaga	L-Vax -- AVAX
laminin A peptides -- NIH	LY 355455 -- Eli Lilly
lanotepase -- Genetics Institute	LY 366405 -- Eli Lilly
laronidase -- BioMarin	LY-355101 -- Eli Lilly
Lassa fever vaccine	Lyme disease DNA vaccine -- Vical/Aventis Pasteur
LCAT -- NIH	Lyme disease vaccine -- Aquila Biopharmaceuticals, Aventis, Pasteur, Symbicom, GlaxoSmithKline, Hyland Immuno, MedImmune
LDP 01 -- Millennium	Lymphocytic choriomeningitis virus vaccine
LDP 02 -- Millennium	lymphoma vaccine -- Biomira, Genitope
Lecithinized superoxide dismutase -- Seikagaku	LYP18
LeIF adjuvant -- Corixa	lys plasminogen, recombinant
leishmaniasis vaccine -- Corixa	Lysosomal storage disease gene therapy -- Avigen
lenercept -- Hoffman La-Roche	lysostaphin -- Nutrition 21
Lenograstim -- Aventis, Chugai	
lepirudin -- Aventis	
leptin -- Amgen, IC Innovations	
Leptin gene therapy -- Chiron Corporation	

FIG. 28Q

## 48/498

M 23 -- Gruenenthal	MEDI 507 -- BioTransplant
M1 monoclonal antibodies -- Acorda	melanin concentrating hormone --
Therapeutics	Neurocrine Biosciences
MA 16N7C2 -- Corvas Intl.	melanocortins -- OMRF
malaria vaccine -- GlaxoSmithKline,	Melanoma monoclonal antibodies -- Viragen
AdProTech, Antigenics, Apovia, Aventis	melanoma vaccine -- GlaxoSmithKline,
Pasteur, Axis Genetics, Behringwerke,	Akzo Nobel, Avant, Aventis Pasteur,
CDCP, Chiron Vaccines, Genzyme	Bavarian Nordic, Biovector, CancerVax,
Transgenics, Hawaii, MedImmune, NIH,	Genzyme Molecular Oncology, Humbolt,
NYU, Oxon, Roche/Saramane, Biotech	ImClone Systems, Memorial, NYU, Oxon
Australia, Rx Tech	Melanoma vaccine Magevac -- Therion
Malaria vaccine CDC/NIIMALVAC-1	memory enhancers -- Scios
malaria vaccine, multicomponent	meningococcal B vaccine -- Chiron
mammaglobin -- Corixa	meningococcal vaccine -- CAMR
mammastatin -- Biotherapeutics	Meningococcal vaccine group B conjugate -
mannan-binding lectin -- NatlImmu	- North American Vaccine
mannan-MUC1 -- Psiron	Meningococcal vaccine group B
MAP 30	recombinant -- BioChem Vaccines,
Marinovir -- Phytera	Microscience
MARstem -- Maret	Meningococcal vaccine group Y conjugate -
MB-015 -- Mochida	- North American Vaccine
MBP -- ImmuLogic	Meningococcal vaccine groups A B and C
MCI-028 -- Mitsubishi-Tokyo	conjugate -- North American Vaccine
MCIF -- Human Genome Sciences	Mepolizumab -- GlaxoSmithKline
MDC -- Advanced BioScience -- Akzo	Metastatin -- EntreMed, Takeda
Nobel, ICOS	Met-CkB7 -- Human Genome Sciences
MDX 11 -- Medarex	met-enkephalin -- TNI
MDX 210 -- Medarex	METH-1 -- Human Genome Sciences
MDX 22 -- Medarex	methioninase -- AntiCancer
MDX 22	Methionine lyase gene therapy --
MDX 240 -- Medarex	AntiCancer
MDX 33	Met-RANTES -- Genexa Biomedical,
MDX 44 -- Medarex	Serono
MDX 447 -- Medarex	Metreleptin
MDX H210 -- Medarex	Microtubule inhibitor MAb
MDX RA -- Houston BioTech., Medarex	Immunogen/Abgenix
ME-104 -- Pharmexa	MGDF -- Kirin
Measles vaccine	MGV -- Progenics
Mecasermin -- Cephalon/Chiron, Chiron	micrin -- Endocrine
MEDI 488 -- MedImmune	microplasmin -- ThromboGenics
MEDI 500	MIF -- Genetics Institute

FIG. 28R

## 49/498

migration inhibitory factor -- NIH	MAb 45-2D9- -- haematoporphyrin conjugate
Mim CD4.1 -- Xycte Therapies	MAb 4B4
mirostipen -- Human Genome Sciences	MAb 4E3-CPA conjugate -- BCM Oncologia
Mitumomab (BEC-2) -- ImClone Systems, Merck KGaA	MAb 4E3-daunorubicin conjugate
MK 852 -- Merck	MAb 50-6
MLN 1202 (Anti-CCR2 monoclonal antibody) -- Millenium Pharmaceuticals	MAb 50-61A -- Institut Pasteur
Mobenakin -- NIS	MAb 5A8 -- Biogen
molgramostim -- Genetics Institute, Novartis	MAb 791T/36-methotrexate conjugate
monoclonal antibodies -- Abgenix/Celltech, Immusol/ Medarex, Viragen/ Roslin Institute, Cambridge Antibody Tech./Elan	MAb 7c11.e8
MAb 108 --	MAb 7E11 C5-selenocystamine conjugate
MAb 10D5 --	MAb 93KA9 -- Novartis
MAb 14.18-interleukin-2 immunocytokine -- Lexigen	MAb A5B7-cisplatin conjugate -- Biodynamics Research, Pharmacia
MAb 14G2a --	MAb A5B7-I-131
MAb 15A10 --	MAb A7
MAb 170 -- Biomira	MAb A717 -- Exocell
MAb 177Lu CC49 --	MAb A7-zinostatin conjugate
MAb 17F9	MAb ABX-RB2 -- Abgenix
MAb 1D7	MAb ACA 11
MAb 1F7 -- Immune Network	MAb AFP-I-131 -- Immunomedics
MAb 1H10-doxorubicin conjugate	MAb AP1
MAb 26-2F	MAb AZ1
MAb 2A11	MAb B3-LysPE40 conjugate
MAb 2E1 -- RW Johnson	MAb B4 -- United Biomedical
MAb 2F5	MAb B43 Genistein-conjugate
MAb 31.1 -- International BioImmune Systems	MAb B43.13-Tc-99m -- Biomira
MAb 32 -- Cambridge Antibody Tech., Peptech	MAb B43-PAP conjugate
MAb 323A3 -- Centocor	MAb B4G7-gelonin conjugate
MAb 3C5	MAb BCM 43-daunorubicin conjugate -- BCM Oncologia
MAb 3F12	MAb BIS-1
MAb 3F8	MAb BMS 181170 -- Bristol-Myers Squibb
MAb 42/6	MAb BR55-2
MAb 425 -- Merck KGaA	MAb BW494
MAb 447-52D -- Merck Sharp & Dohme	MAb C 242-DM1 conjugate -- ImmunoGen
	MAb C242-PE conjugate
	MAb c30-6
	MAb CA208-cytorhodin-S conjugate -- Hoechst Japan
	MAb CC49 -- Enzon

FIG. 28S



## 50/498

MAb ch14.18 –	MAb LL2-I-131 – Immunomedics
MAb CH14.18-GM-CSF fusion protein --	MAb LL2-Y-90
Lexigen	MAb LS2D617 -- Hybritech
MAb chCE7	MAb LYM-1-gelonin conjugate
MAb CI-137 – AMRAD	MAb LYM-1-I-131
MAb cisplatin conjugate	MAb LYM-1-Y-90
MAb CLB-CD19	MAb LYM-2 -- Peregrine
MAb CLB-CD19v	MAb M195
MAb CLL-1 -- Peregrine	MAb M195-bismuth 213 conjugate --
MAb CLL-1-GM-CSF conjugate	Protein Design Labs
MAb CLL-1-IL-2 conjugate -- Peregrine	MAb M195-gelonin conjugate
MAb CLN IgG -- doxorubicin conjugates	MAb M195-I-131
MAb conjugates – Tanox	MAb M195-Y-90
MAb D612	MAb MA 33H1 -- Sanofi
MAb Dal B02	MAb MAD11
MAb DC101 -- ImClone	MAb MGb2
MAb EA 1 –	MAb MINT5
MAb EC708 -- Biovation	MAb MK2-23
MAb EP-5C7 -- Protein Design Labs	MAb MOC31 ETA(252-613) conjugate
MAb ERIC-1 -- ICRT	MAb MOC-31-In-111
MAb F105 gene therapy	MAb MOC-31-PE conjugate
MAb FC 2.15	MAb MR6 –
MAb G250 -- Centocor	MAb MRK-16 -- Aventis Pasteur
MAb GA6	MAb MS11G6
MAb GA733	MAb MX-DTPA BrE-3
MAb Gliomab-H -- Viventia Biotech	MAb MY9
MAb HB2-saporin conjugate	MAb Nd2 -- Tosoh
MAb HD 37 –	MAb NG-1 -- Hygeia
MAb HD37-ricin chain-A conjugate	MAb NM01 – Nissin Food
MAb HNK20 – Acambis	MAb OC 125
MAb huN901-DM1 conjugate --	MAb OC 125-CMA conjugate
ImmunoGen	MAb OKI-1 -- Ortho-McNeil
MAb I-131 CC49 -- Corixa	MAb OX52 -- Bioproducts for Science
MAb ICO25	MAb PMA5
MAb ICR12-CPG2 conjugate	MAb PR1
MAb ICR-62	MAb prost 30
MAb IRac-ricin A conjugate	MAb R-24
MAb K1	MAb R-24 $\alpha$ Human GD3 – Celltech
MAb KS1-4-methotrexate conjugate	MAb RFB4-ricin chain A conjugate
MAb L6 -- Bristol-Myers Squibb, Oncogen	MAb RFT5-ricin chain A conjugate
MAb LiCO 16-88	MAb SC 1

FIG. 28T

## 51/498

MAB SM-3 -- ICRT	Muc-1 vaccine -- Corixa
MAB SMART 1D10 -- Protein Design Labs	mucosal tolerance -- Aberdeen
MAB SMART ABL 364 -- Novartis	müllerian inhibiting subst
MAB SN6f	muplestim -- Genetics Institute, Novartis,
MAB SN6f-deglycosylated ricin A chain	DSM Anti-Infectives
conjugate --	murine MAB -- KS Biomedix
MAB SN6j	Mutant somatropin -- JCR Pharmaceutical
MAB SN7-ricin chain A conjugate	MV 833 -- Toagosei
MAB T101-Y-90 conjugate -- Hybritech	Mycoplasma pulmonis vaccine
MAB T-88 -- Chiron	Mycoprex -- XOMA
MAB TB94 -- Cancer ImmunoBiology	myeloperoxidase -- Henogen
MAB TEC 11	myostatin -- Genetics Institute
MAB TES-23 -- Chugai	Nacolomab tafenatox -- Pharmacia
MAB TM31 -- Avant	Nagrecor -- Scios
MAB TNT-1 -- Cambridge Antibody Tech.,	nagrestipen -- British Biotech
Peregrine	NAP-5 -- Corvas Intl.
MAB TNT-3	NAPc2 -- Corvas Intl.
MAB TNT-3 -- IL2 fusion protein --	nartograstim -- Kyowa
MAB TP3-At-211	Natalizumab -- Protein Design Labs
MAB TP3-PAP conjugate --	Nateplase -- NIH, Nihon Schering
MAB UJ13A -- ICRT	nateplase -- Schering AG
MAB UN3	NBI-3001 -- Neurocrine Biosci.
MAB ZME-018-gelonin conjugate	NBI-5788 -- Neurocrine Biosci.
MAB-BC2 -- GlaxoSmithKline	NBI-6024 -- Neurocrine Biosci.
MAB-DM1 conjugate -- ImmunoGen	Nef inhibitors -- BRI
MAB-ricin-chain-A conjugate -- XOMA	Neisseria gonorrhoea vaccine -- Antex
MAB-temoporfin conjugates	Biologics
Monopharm C -- Viventia Biotech	Neomycin B-arginine conjugate
monteplase -- Eisai	Nerelimomab -- Chiron
montirelin hydrate -- Gruenenthal	Nerve growth factor -- Amgen -- Chiron,
moroctocog alfa -- Genetics Institute	Genentech
Moroctocog-alfa -- Pharmacia	Nerve growth factor gene therapy
MP 4	nesiritide citrate -- Scios
MP-121 -- Biopharm	neuregulin-2 -- CeNeS
MP-52 -- Biopharm	neurocan -- NYU
MRA -- Chugai	neuronal delivery system -- CAMR
MS 28168 -- Mitsui Chemicals, Nihon	Neurophil inhibitory Factor -- Corvas
Schering	Neuroprotective vaccine -- University of
MSH fusion toxin -- Ligand	Auckland
MSI-99 -- Genaera	neurotrophic chimaeras -- Regeneron
MT 201 -- Micromet	neurotrophic factor -- NsGene, CereMedix

FIG. 28U

52/498

NeuroVax -- Immune Response	Oncophage -- Antigenics
neurturin -- Genentech	Oncostatin M -- Bristol-Myers Squibb
neutral endopeptidase -- Genentech	OncoVax-CL -- Jenner Biotherapies
NGF enhancers -- NeuroSearch	OncoVax-P -- Jenner Biotherapies
NHL vaccine -- Large Scale Biology	onercept -- Yeda
NIP45 -- Boston Life Sciences	onychomycosis vaccine -- Boehringer
NKI-B20	Ingelheim
NM 01 -- Nissin Food	opebecan -- XOMA
NMI-139 -- NitroMed	opioids -- Arizona
NMMP -- Genetics Institute	Oprelvekin -- Genetics Institute
NN-2211 -- Novo Nordisk	Oregovomab -- AltaRex
Noggin -- Regeneron	Org-33408 b-- Akzo Nobel
Nonacog alfa	Orolip DP -- EpiCept
Norelin -- Biostar	oryzacystatin
Norwalk virus vaccine	OSA peptides -- GenSci Regeneration
NRLU 10 -- NeoRx	osteoblast-cadherin GF -- Pharis
NRLU 10 PE -- NeoRx	Osteocalcin-thymidine kinase gene therapy
NT-3 -- Regeneron	osteogenic protein -- Curis
NT-4/5 -- Genentech	osteopontin -- OraPharma
NU 3056	osteoporosis peptides -- Integra, Telios
NU 3076	osteoprotegerin -- Amgen, SnowBrand
NX 1838 -- Gilead Sciences	otitis media vaccines -- Antex Biologics
NY ESO-1/CAG-3 antigen -- NIH	ovarian cancer -- University of Alabama
NYVAC-7 -- Aventis Pasteur	OX40-IgG fusion protein -- Cantab, Xenova
NZ-1002 -- Novazyme	P 246 -- Diatide
obesity therapy -- Nobex	P 30 -- Alfacell
OC 10426 -- Ontogen	p1025 -- Active Biotech
OC 144093 -- Ontogen	P-113 <sup>^</sup> -- Demegen
OCIF -- Sankyo	P-16 peptide -- Transition Therapeutics
Oct-43 -- Otsuka	p43 -- Ramot
Odulimomab -- Immunotech	P-50 peptide -- Transition Therapeutics
OK PSA - liposomal	p53 + RAS vaccine -- NIH, NCI
OKT3-gamma-1-ala-ala	PACAP(1-27) analogue
OM 991	paediatric vaccines -- Chiron
OM 992	Pafase -- ICOS
Omalizumab -- Genentech	PAGE-4 plasmid DNA -- IDEC
oncoimmunin-L -- NIH	PAI-2 -- Biotech Australia, Human
Oncolysin B -- ImmunoGen	Therapeutics
Oncolysin CD6 -- ImmunoGen	Palifermin (keratinocyte growth factor) --
Oncolysin M -- ImmunoGen	Amgen
Oncolysin S -- ImmunoGen	Palivizumab -- MedImmune

FIG. 28V

## 53/498

PAM 4 -- Merck	PEG-uricase -- Mountain View
paniteplase -- Yamanouchi	Pegvisomant -- Genentech
pancreatin, Minitabs -- Eurand	PEGylated proteins, PolyMASC -- Valentis
Pangen -- Fournier	PEGylated recombinant native human leptin
Pantarin -- Selective Genetics	-- Roche
Parainfluenza virus vaccine -- Pharmacia,	Pemtumomab
Pierre Fabre	Penetratin -- Cyclacel
paraoxanase -- Esperion	Pepscan -- Antisoma
parathyroid hormone -- Abiogen, Korea	peptide G -- Peptech, ICRT
Green Cross	peptide vaccine -- NIH, NCI
Parathyroid hormone (1-34) --	Pexelizumab
Chugai/Suntory	pexiganan acetate -- Genaera
Parkinson's disease gene therapy -- Cell	Pharmaprojects No. 3179 -- NYU
Genesys/ Ceregene	Pharmaprojects No. 3390 -- Ernest Orlando
Parvovirus vaccine -- MedImmune	Pharmaprojects No. 3417 -- Sumitomo
PCP-Scan -- Immunomedics	Pharmaprojects No. 3777 -- Acambis
PDGF -- Chiron	Pharmaprojects No. 4209 -- XOMA
PDGF cocktail -- Theratechnologies	Pharmaprojects No. 4349 -- Baxter Intl.
peanut allergy therapy -- Dynavax	Pharmaprojects No. 4651
PEG anti-ICAM MAb -- Boehringer	Pharmaprojects No. 4915 -- Avanir
Ingelheim	Pharmaprojects No. 5156 -- Rhizogenics
PEG asparaginase -- Enzon	Pharmaprojects No. 5200 -- Pfizer
PEG glucocerebrosidase	Pharmaprojects No. 5215 -- Origene
PEG hirudin -- Knoll	Pharmaprojects No. 5216 -- Origene
PEG interferon-alpha-2a -- Roche	Pharmaprojects No. 5218 -- Origene
PEG interferon-alpha-2b + ribavirin --	Pharmaprojects No. 5267 -- ML
Biogen, Enzon, ICN Pharmaceuticals,	Laboratories
Schering-Plough	Pharmaprojects No. 5373 -- MorphoSys
PEG MAb A5B7 --	Pharmaprojects No. 5493 -- Metabolex
Pegacaristim -- Amgen -- Kirin Brewery --	Pharmaprojects No. 5707 -- Genentech
ZymoGenetics	Pharmaprojects No. 5728 -- Autogen
Pegaldesleukin -- Research Corp	Pharmaprojects No. 5733 -- BioMarin
pegaspargase -- Enzon	Pharmaprojects No. 5757 -- NIH
pegfilgrastim -- Amgen	Pharmaprojects No. 5765 -- Gryphon
PEG-interferon Alpha -- Viragen	Pharmaprojects No. 5830 -- AntiCancer
PEG-interferon Alpha 2A -- Hoffman La-	Pharmaprojects No. 5839 -- Dyax
Roche	Pharmaprojects No. 5849 -- Johnson &
PEG-interferon Alpha 2B -- Schering-	Johnson
Plough	Pharmaprojects No. 5860 -- Mitsubishi-
PEG-r-hirudin -- Abbott	Tokyo
PEG-rHuMGDF -- Amgen	

FIG. 28W

54/498

Pharmaprojects No. 5869 -- Oxford GlycoSciences	Plasminogen activators -- Abbott Laboratories, American Home Products,
Pharmaprojects No. 5883 -- Asahi Brewery	Boehringer Mannheim, Chiron
Pharmaprojects No. 5947 -- StressGen	Corporation, DuPont Pharmaceuticals, Eli Lilly, Shionogi, Genentech, Genetics
Pharmaprojects No. 5961 -- Theratechnologies	Institute, GlaxoSmithKline, Hemispherx
Pharmaprojects No. 5962 -- NIH	Biopharma, Merck & Co, Novartis,
Pharmaprojects No. 5966 -- NIH	Pharmacia Corporation, Wakamoto, Yeda
Pharmaprojects No. 5994 -- Pharming	plasminogen-related peptides -- Bio-Tech.
Pharmaprojects No. 5995 -- Pharming	General/MGH
Pharmaprojects No. 6023 -- IMMUCON	platelet factor 4 -- RepliGen
Pharmaprojects No. 6063 -- Cytoclonal	Platelet-derived growth factor -- Amgen --
Pharmaprojects No. 6073 -- SIDDCO	ZymoGenetics
Pharmaprojects No. 6115 -- Genzyme	plusonemin-- Hayashibara
Pharmaprojects No. 6227 -- NIH	PMD-2850 -- Protherics
Pharmaprojects No. 6230 -- NIH	Pneumococcal vaccine -- Antex Biologics,
Pharmaprojects No. 6236 -- NIH	Aventis Pasteur
Pharmaprojects No. 6243 -- NIH	Pneumococcal vaccine intranasal --
Pharmaprojects No. 6244 -- NIH	BioChem Vaccines/Biovector
Pharmaprojects No. 6281 -- Senetek	PR1A3
Pharmaprojects No. 6365 -- NIH	PR-39
Pharmaprojects No. 6368 -- NIH	pralmorelin -- Kaken
Pharmaprojects No. 6373 -- NIH	Pretarget-Lymphoma -- NeoRx
Pharmaprojects No. 6408 -- Pan Pacific	Priliximab -- Centocor
Pharmaprojects No. 6410 -- Athersys	PRO 140 -- Progenics
Pharmaprojects No. 6421 -- Oxford GlycoSciences	PRO 2000 -- Procept
Pharmaprojects No. 6522 -- Maxygen	PRO 367 -- Progenics
Pharmaprojects No. 6523 -- Pharis	PRO 542 -- Progenics
Pharmaprojects No. 6538 -- Maxygen	pro-Apo A-I -- Esperion
Pharmaprojects No. 6554 -- APALEXO	prolactin -- Genzyme
Pharmaprojects No. 6560 -- Ardana	Prosaptide TX14(A) -- Bio-Tech. General
Pharmaprojects No. 6562 -- Bayer	prostate cancer antibodies -- Immunex,
Pharmaprojects No. 6569 -- Eos	UroCor
Phenoxazine	prostate cancer antibody therapy --
Phenylase -- Ibex	Genentech/UroGenesys,
Pigment epithelium derived factor --	Genotherapeutics
plasminogen activator inhibitor-1,	prostate cancer immunotherapeutics -- The
recombinant -- DuPont Pharmaceuticals	PSMA Development Company
	prostate cancer vaccine -- Aventis Pasteur,
	Zonagen, Corixa, Dendreon, Jenner
	Biotherapies, Therion Biologics

FIG. 28X

55/498

prostate-specific antigen -- EntreMed	RD 62198
protein A -- RepliGen	rDnase -- Genentech
protein adhesives -- Enzon	RDP-58 -- SangStat
protein C -- Baxter Intl., PPL Therapeutics,	RecepTox-Fce -- Keryx
ZymoGenetics	RecepTox-GnRH -- Keryx, MTR
protein C activator -- Gilead Sciences	Technologies
protein kinase R antags -- NIH	RecepTox-MBP -- Keryx, MTR
protirelin -- Takeda	Technologies
protocadherin 2 -- Caprion	recFSH -- Akzo Nobel, Organon
Pro-urokinase -- Abbott, Bristol-Myers	REGA 3G12
Squibb, Dainippon, Tosoh -- Welfide	Regavirumab -- Teijin
P-selectin glycoprotein ligand-1 -- Genetics	relaxin -- Connetics Corp
Institute	Renal cancer vaccine -- Macropharm
pseudomonal infections -- InterMune	repifermin -- Human Genome Sciences
Pseudomonas vaccine -- Cytovax	Respiratory syncytial virus PFP-2 vaccine --
PSGL-Ig -- American Home Products	Wyeth-Lederle
PSP-94 -- Procyon	Respiratory syncytial virus vaccine --
PTH 1-34 -- Nobex	GlaxoSmithKline, Pharmacia, Pierre Fabre
Quilimmune-M -- Antigenics	Respiratory syncytial virus vaccine
R 744 -- Roche	inactivated
R 101933	Respiratory syncytial virus-parainfluenza
R 125224 -- Sankyo	virus vaccine -- Aventis Pasteur,
RA therapy -- Cardion	Pharmacia
Rabies vaccine recombinant -- Aventis	Retepase -- Boehringer Mannheim,
Pasteur, BioChem Vaccines, Kaketsuken	Hoffman La-Roche
Pharmaceuticals	Retropep -- Retroscreen
RadioTheraCIM -- YM BioSciences	RFB4 (dsFv) PE38
Ramot project No. 1315 -- Ramot	RFI 641 -- American Home Products
Ramot project No. K-734A -- Ramot	RFTS -- UAB Research Foundation
Ramot project No. K-734B -- Ramot	RG 12986 -- Aventis Pasteur
Ranibizumab (Anti-VEGF fragment) --	RG 83852 -- Aventis Pasteur
Genentech	RG-1059 -- RepliGen
RANK -- Immunex	rGCR -- NIH
ranpirnase -- Alfacell	rGLP-1 -- Restoragen
ranpirnase-anti-CD22 MAb -- Alfacell	rGRF -- Restoragen
RANTES inhibitor -- Milan	rh Insulin -- Eli Lilly
RAPID drug delivery systems -- ARIAD	RHAMM targeting peptides -- Cangene
rasburicase -- Sanofi	rHb1.1 -- Baxter Intl.
rBPI-21, topical -- XOMA	rhCC10 -- Claragen
RC 529 -- Corixa	rhCG -- Serono
rCFTR -- Genzyme Transgenics	Rheumatoid arthritis gene therapy

FIG. 28Y

56/498

Rheumatoid arthritis vaccine -- Veterans Affairs Medical Center	SB RA 31012 --
rhLH -- Serono	SC 56929 -- Pharmacia
Ribozyme gene therapy -- Genset	SCA binding proteins -- Curis, Enzon
Rickettsial vaccine recombinant	scFv(14E1)-ETA Berlex Laboratories, Schering AG
RIGScan CR -- Neoprobe	ScFv(FRP5)-ETA --
RIP-3 -- Rigel	ScFv6C6-PE40 --
Rituximab -- Genentech	SCH 55700 -- Celltech
RK-0202 -- RxKinetix	Schistosomiasis vaccine -- Glaxo Wellcome/Medevea, Brazil
RLT peptide -- Esperion	SCPF -- Advanced Tissue Sciences
rM/NEI -- IVAX	scuPA-suPAR complex -- Hadasit
rmCRP -- Immtech	SD-9427 -- Pharmacia
RN-1001 -- Renovo	SDF-1 -- Ono
RN-3 -- Renovo	SDZ 215918 -- Novartis
RNAse conjugate -- Immunomedics	SDZ 280125 -- Novartis
RO 631908 -- Roche	SDZ 89104 -- Novartis
Rotavirus vaccine -- Merck	SDZ ABL 364 -- Novartis
RP 431 -- DuPont Pharmaceuticals	SDZ MMA 383 -- Novartis
RP-128 -- Resolution	Secretin -- Ferring, Repligen
RPE65 gene therapy --	serine protease inhbs -- Pharis
RPR 110173 -- Aventis Pasteur	sermorelin acetate -- Serono
RPR 115135 -- Aventis Pasteur	SERP-1 -- Viron
RPR 116258A -- Aventis Pasteur	sertenef -- Dainippon
rPSGL-Ig -- American Home Products	serum albumin, Recombinant human -- Aventis Behring
r-SPC surfactant -- Byk Gulden	serum-derived factor -- Hadasit
RSV antibody -- Medimmune	Sevirumab -- Novartis
Ruplizumab -- Biogen	SGN 14 -- Seattle Genetics
rV-HER-2/neu -- Therion Biologics	SGN 15 -- Seattle Genetics
SA 1042 -- Sankyo	SGN 17/19 -- Seattle Genetics
sacrosidase -- Orphan Medical	SGN 30 -- Seattle Genetics
Sant 7	SGN-10 -- Seattle Genetics
Sargramostim -- Immunex	SGN-11 -- Seattle Genetics
saruplase -- Gruenenthal	SH 306 -- DuPont Pharmaceuticals
Satumomab -- Cytogen	Shanvac-B -- Shantha
SB 1 -- COR Therapeutics	Shigella flexneri vaccine -- Avant, Acambis, Novavax
SB 207448 -- GlaxoSmithKline	Shigella sonnei vaccine --
SB 208651 -- GlaxoSmithKline	sICAM-1 -- Boehringer Ingelheim
SB 240683 -- GlaxoSmithKline	Silteplase -- Genzyme
SB 249415 -- GlaxoSmithKline	
SB 249417 -- GlaxoSmithKline	
SB 6 -- COR Therapeutics	

FIG. 28Z

57/498

SIV vaccine -- Endocon, Institut Pasteur	Staphylococcus aureus vaccine conjugate --
SK 896 -- Sanwa Kagaku Kenkyusho	Nabi
SK-827 -- Sanwa Kagaku Kenkyusho	Staphylococcus therapy -- Tripep
Skeletex -- CellFactors	Staphylokinase -- Biovation, Prothera,
SKF 106160 -- GlaxoSmithKline	Thrombogenetics
S-nitroso-AR545C --	Streptococcal A vaccine -- M6
SNTP -- Active Biotech	Pharmaceuticals, North American Vaccine
somatomedin-1 -- GroPep, Mitsubishi-	Streptococcal B vaccine -- Microscience
Tokyo, NIH	Streptococcal B vaccine recombinant --
somatomedin-1 carrier protein -- Insmed	Biochem Vaccines
somatostatin -- Ferring	Streptococcus pyogenes vaccine
Somatotropin/	STRL-33 -- NIH
Human Growth Hormone -- Bio-Tech.	Subalin -- SRC VB VECTOR
General, Eli Lilly	SUIS -- United Biomedical
somatropin -- Bio-Tech. General, Alkermes,	SUIS-LHRH -- United Biomedical
ProLease, Aventis Behring, Biovector,	SUN-E3001 -- Suntory
Cangene, Dong-A, Eli Lilly, Emisphere,	super high affinity monoclonal antibodies --
Enact, Genentech, Genzyme Transgenics,	YM BioSciences
Grandis/InfiMed, CSL, InfiMed, MacroMed,	Superoxide dismutase -- Chiron, Enzon,
Novartis, Novo Nordisk, Pharmacia	Ube Industries, Bio-Tech, Yeda
Serono, TranXenoGen	superoxide dismutase-2 -- OXIS
somatropin derivative -- Schering AG	suppressin -- UAB Research Foundation
somatropin, AIR -- Eli Lilly	SY-161-P5 -- ThromboGenics
Somatropin, inhaled -- Eli Lilly/Alkermes	SY-162 -- ThromboGenics
somatropin, Kabi -- Pharmacia	Systemic lupus erythematosus vaccine --
somatropin, Orasome -- Novo Nordisk	MedClone/VivoRx
Sonermin -- Daiippon Pharmaceutical	T cell receptor peptides -- Xoma
SP(V5.2)C -- Supertek	T cell receptor peptide vaccine
SPf66	T4N5 liposomes -- AGI Dermatics
sphingomyelinase -- Genzyme	TACI, soluble -- ZymoGenetics
SR 29001 -- Sanofi	targeted apoptosis -- Antisoma
SR 41476 -- Sanofi	tasonermin -- Boehringer Ingelheim
SR-29001 -- Sanofi	TASP
SS1(dsFV)-PE38 -- NeoPharm	TASP-V
$\beta$ 2 microglobulin -- Avidex	Tat peptide analogues -- NIH
$\beta$ 2-microglobulin fusion proteins -- NIH	TBP I -- Yeda
$\beta$ -amyloid peptides -- CeNeS	TBP II
$\beta$ -defensin -- Pharis	TBV25H -- NIH
Staphylococcus aureus infections --	Tc 99m ior cea1 -- Center of Molecular
Inhibitex/ZLB	Immunology
	Tc 99m P 748 -- Diatide

FIG. 28AA



58/498

Tc 99m votumumab -- Intracell	Tissue factor -- Genentech
Tc-99m rh-Annexin V -- Theseus Imaging	Tissue factor pathway inhibitor
teceleukin -- Biogen	TJN-135 -- Tsumura
tenecteplase -- Genentech	TM 27 -- Avant
Teriparatide -- Armour Pharmaceuticals,	TM 29 -- Avant
Asahi Kasei, Eli Lilly	TMC-151 -- Tanabe Seiyaku
terlipressin -- Ferring	TNF tumour necrosis factor -- Asahi Kasei
testisin -- AMRAD	TNF Alpha -- CytImmune
Tetrafibricin -- Roche	TNF antibody -- Johnson & Johnson
TFPI -- EntreMed	TNF binding protein -- Amgen
tgD-IL-2 -- Takeda	TNF degradation product -- Oncotech
TGF-Alpha -- ZymoGenetics	TNF receptor -- Immunex
TGF- $\beta$ -- Kolon	TNF receptor 1, soluble -- Amgen
TGF- $\beta$ 2 -- Insmed	TNF Tumour necrosis factor-alpha -- Asahi
TGF- $\beta$ 3 -- OSI	Kasei, Genetech, Mochida
Thalassaemia gene therapy -- Crucell	TNF-Alpha inhibitor -- Tripep
TheraCIM-h-R3 -- Center of Molecular	TNFR:Fc gene therapy -- Targeted Genetics
Immunology, YM BioSciences	TNF-SAM2
Theradigm-HBV -- Epimmune	Tolerimab -- Innogenetics
Theradigm-HPV -- Epimmune	Toxoplasma gondii vaccine --
Theradigm-malaria -- Epimmune	GlaxoSmithKline
Theradigm-melanoma -- Epimmune	TP 9201 -- Telios
TheraFab -- Antisoma	TP10 -- Avant
ThGRF 1-29 -- Theratechnologies	TP20 -- Avant
ThGRF 1-44 -- Theratechnologies	tPA -- Centocor
Thrombin receptor activating peptide --	trafermin -- Scios
Abbott	TRAIL/Apo2L -- Immunex
thrombomodulin -- Iowa, Novocastra	TRAIL-R1 MAb -- Cambridge Antibody
Thrombopoietin -- Dragon Pharmaceuticals,	Technologies
Genentech	transferrin-binding proteins -- CAMR
thrombopoietin, Pliva -- Recepton	Transforming growth factor-beta-1 --
Thrombospondin 2 --	Genentech
thrombostatin -- Thromgen	transport protein -- Genesis
thymalfasin -- SciClone	Trastuzumab -- Genetech
thymocartin -- Gedeon Richter	TRH -- Ferring
thymosin Alpha1 -- NIH	Triabin -- Schering AG
thyroid stimulating hormone -- Genzyme	Triconal
tlCAM-1 -- Bayer	Triflavin
Tick anticoagulant peptide -- Merck	troponin I -- Boston Life Sciences
TIF -- Xoma	TRP-2 <sup>A</sup> -- NIH
Tifacogin -- Chiron, NIS, Pharmacia	trypsin inhibitor -- Mochida

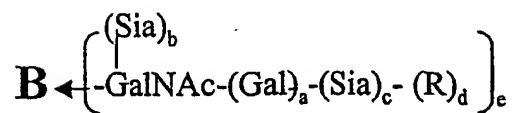
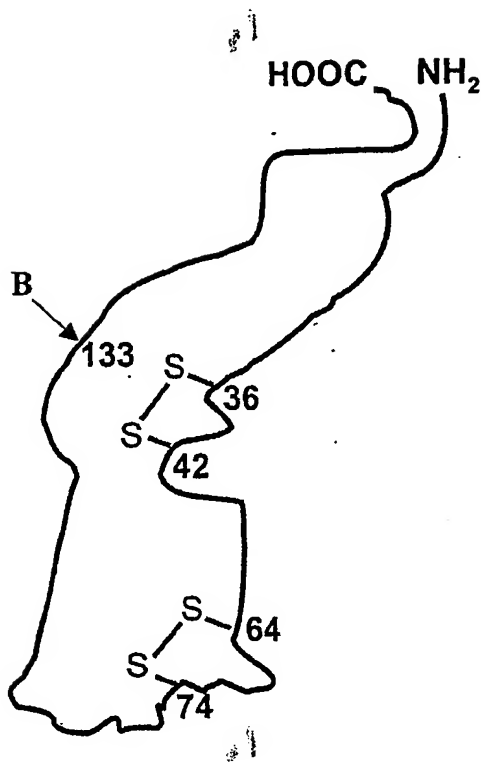
FIG. 28BB

59/498

TSP-1 gene therapy –	Vascular endothelial growth factors – R&D
TT-232	Systems
TTS-CD2 -- Active Biotech	vascular targeting agents -- Peregrine
Tuberculosis vaccine -- Aventis Pasteur,	vasopermeation enhancement agents --
Genesis	Peregrine
Tumor Targeted Superantigens -- Active	vasostatin -- NIH
Biotech -- Pharmacia	VCL -- Bio-Tech. General
tumour vaccines -- PhotoCure	VEGF -- Genentech, Scios
tumour-activated prodrug antibody	VEGF inhibitor -- Chugai
conjugates -- Millennium/ImmunoGen	VEGF-2 -- Human Genome Sciences
tumstatin -- ILEX	VEGF-Trap -- Regeneron
Tuvirumab -- Novartis	viscumin, recombinant -- Madaus
TV-4710 -- Teva	Vitaxin
TWEAK receptor -- Immunex	Vitrax -- ISTA Pharmaceuticals
TXU-PAP	West Nile virus vaccine -- Bavarian Nordic
TY-10721 -- TOA Eiyo	WP 652
Type I diabetes vaccine -- Research Corp	WT1 vaccine -- Corixa
Typhoid vaccine CVD 908	WX-293 -- Willex BioTech.
U 143677 -- Pharmacia	WX-360 -- Willex BioTech.
U 81749 -- Pharmacia	WX-UK1 -- Willex BioTech.
UA 1248 -- Arizona	XMP-500 -- XOMA
UGIF -- Sheffield	XomaZyme-791 -- XOMA
UIC 2	XTL 001 -- XTL Biopharmaceuticals
UK 101	XTL 002 -- XTL Biopharmaceuticals
UK-279276 -- Corvas Intl.	yeast delivery system -- Globelimmune
urodilatin -- Pharis	Yersinia pestis vaccine
urofolitrophin -- Serono	YIGSR-Stealth -- Johnson & Johnson
Urokinase -- Abbott	Yisum Project No. D-0460 -- Yisum
uteroferrin -- Pepgen	YM 207 -- Yamanouchi
V 20 -- GLYCODESIGN	YM 337 -- Protein Design Labs
V2 vasopressin receptor gene therapy	Yttrium-90 labelled biotin
vaccines -- Active Biotech	Yttrium-90-labeled anti-CEA MAb T84.66 --
Varicella zoster glycoprotein vaccine --	ZD 0490 -- AstraZeneca
Research Corporation Technologies	ziconotide -- Elan
Varicella zoster virus vaccine live -- Cantab	ZK 157138 -- Berlex Laboratories
Pharmaceuticals	Zolimomab aritox
Vascular endothelial growth factor --	Zorcell -- Immune Response
Genentech, University of California	ZRXL peptides -- Novartis

FIG. 28CC

60/498



a-c, e (independently selected) = 0 or 1;

d = 0;

R = modifying group, sialyl or oligosialyl

FIG. 29A

61/498

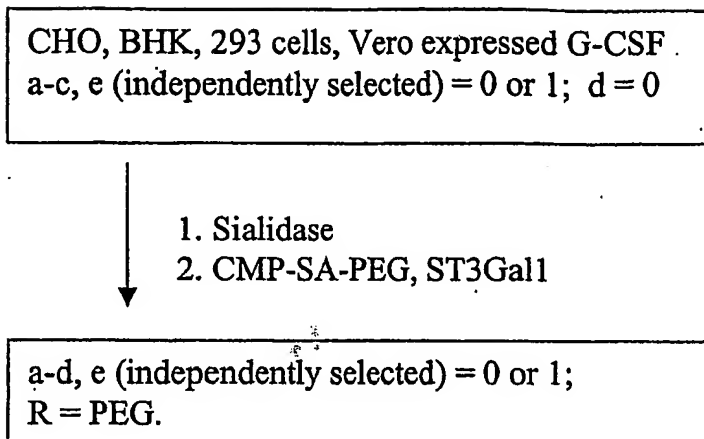


FIG. 29B

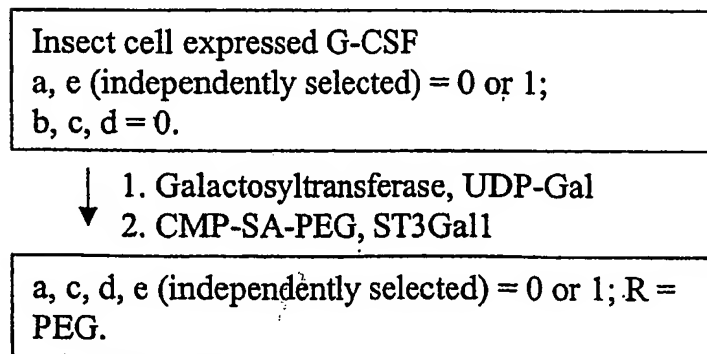


FIG. 29C

62/498

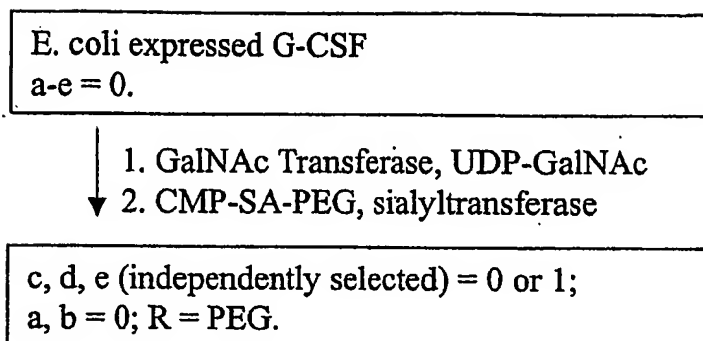


FIG. 29D

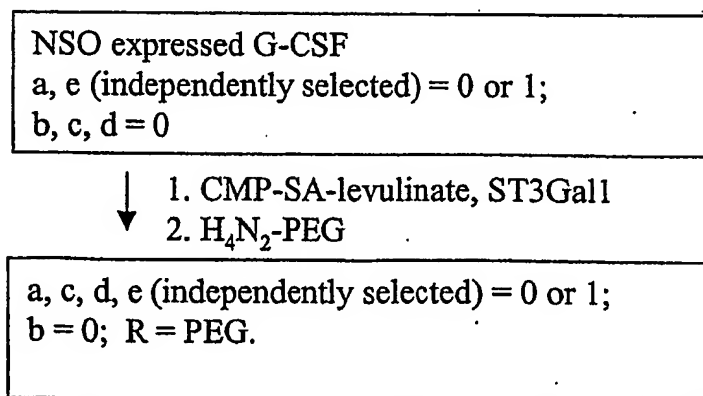


FIG. 29E

63/498

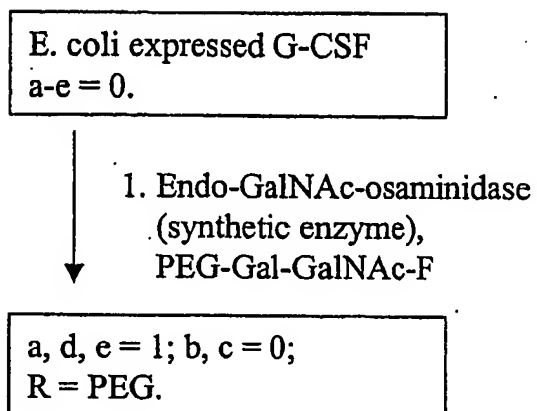


FIG. 29F

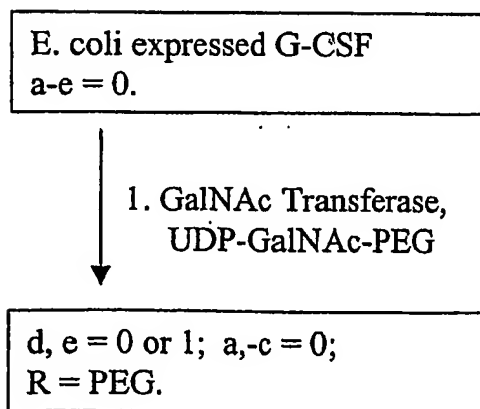
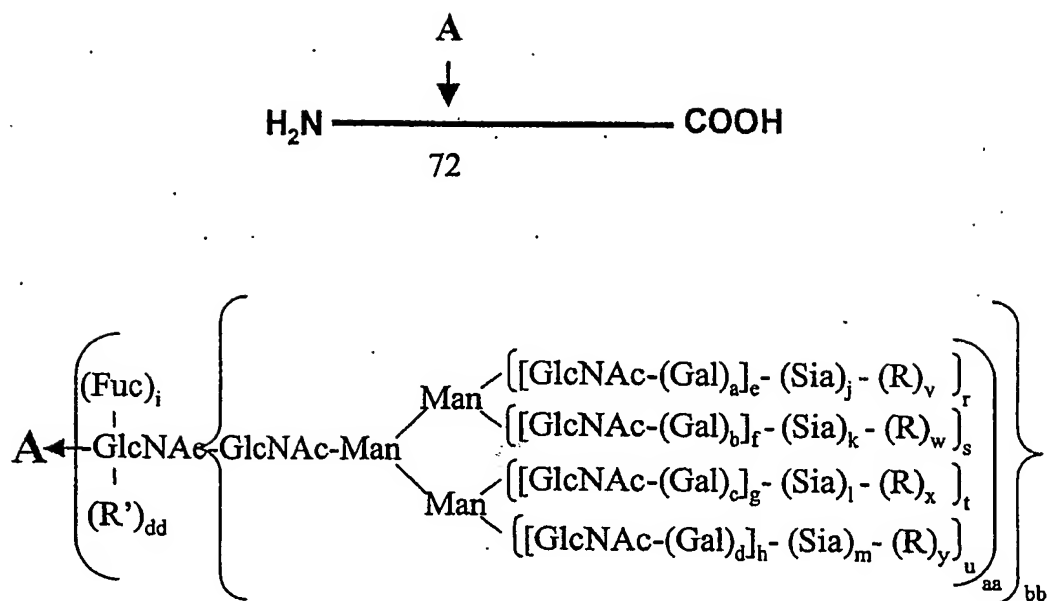


FIG. 29G

64/498



a-d, i, n-u (independently selected) = 0 or 1.

aa, bb, cc, dd, ee (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 20.

v-z = 0; R = modifying group, mannose, oligo-mannose.

R' = H, glycosyl residue, modifying group,  
glycoconjugate.

FIG. 30A

65/498

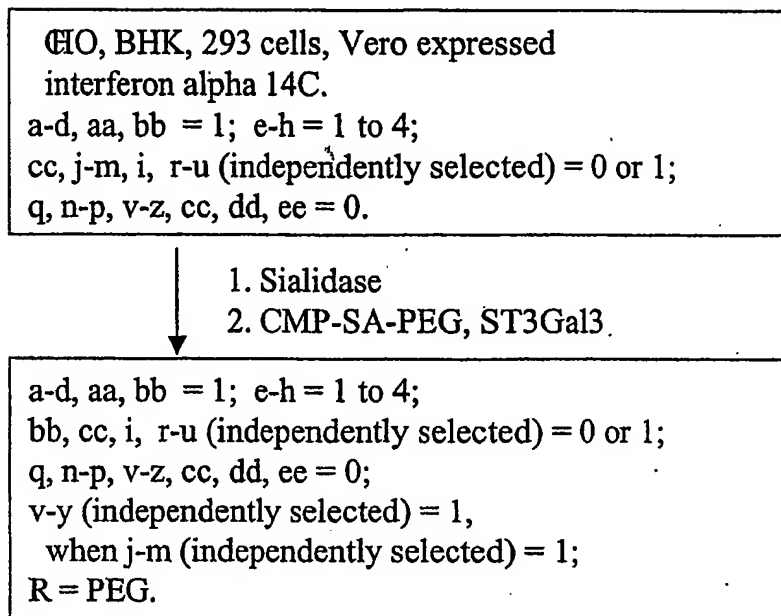


FIG. 30B

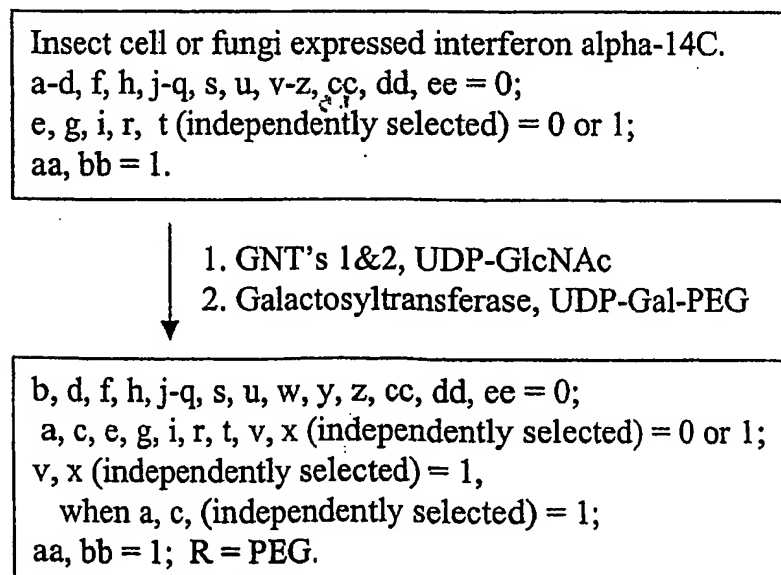


FIG. 30C



66/498

Yeast expressed interferon alpha-14C.

a-q, cc, dd, ee, v-z = 0;

r-y (independently selected) = 0 to 1;

aa, bb = 1;

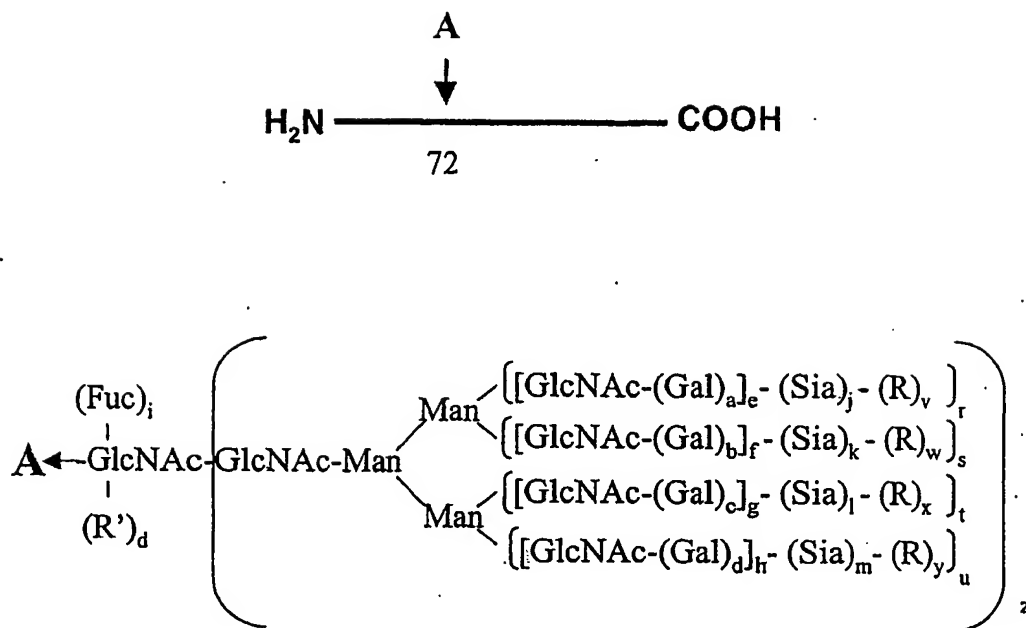
R (branched or linear) = Man, oligomannose or polysaccharide.

- ↓
1. Endo-H
  2. Galactosyltransferase, UDP-Gal
  - 3.. CMP-SA-PEG, ST3Gal3

a-z, bb = 0; aa = 1; R' = -Gal-Sia-PEG.

FIG. 30D

67/498



a-d, i, r-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 or 1.

n, v-y = 0; z = 0 or 1.

R = polymer; R' = sugar, glycoconjugate.

FIG. 30E

68/498

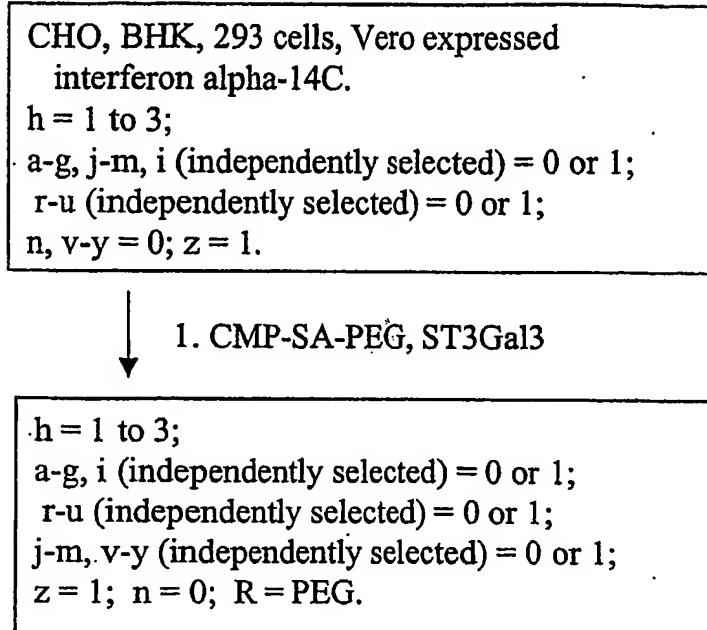


FIG. 30F

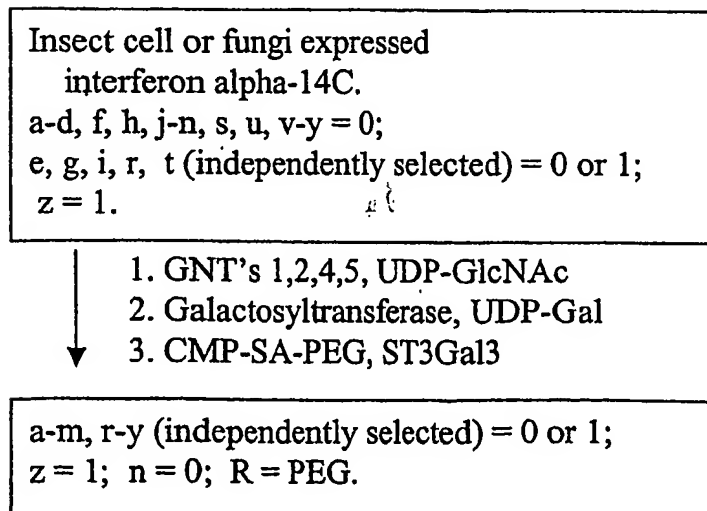


FIG. 30G

69/498

Yeast expressed interferon alpha-14C.  
 a-n = 0; r-y (independently selected) = 0 to 1;  
 z = 1; R (branched or linear) = Man,  
 oligomannose.

1. mannosidases
2. GNT's 1,2,4,5, UDP-GlcNAc
3. Galactosyltransferase, UDP-Gal
4. CMP-SA-PEG, ST3Gal3

a-m, r-y (independently selected) = 0 or 1;  
 z = 1; n = 0; R = PEG.

FIG. 30H

NSO expressed interferon alpha 14C.  
 a-i, r-u (independently selected) = 0 or 1;  
 j-m, n, v-y = 0; z = 1.

1. CMP-SA-levulinate, ST3Gal3,  
buffer, salt
2. H<sub>4</sub>N<sub>2</sub>-PEG

a-i, j-m, r-y (independently selected) = 0 or 1;  
 n = 0; z = 1; R = PEG.

FIG. 30I

70/498

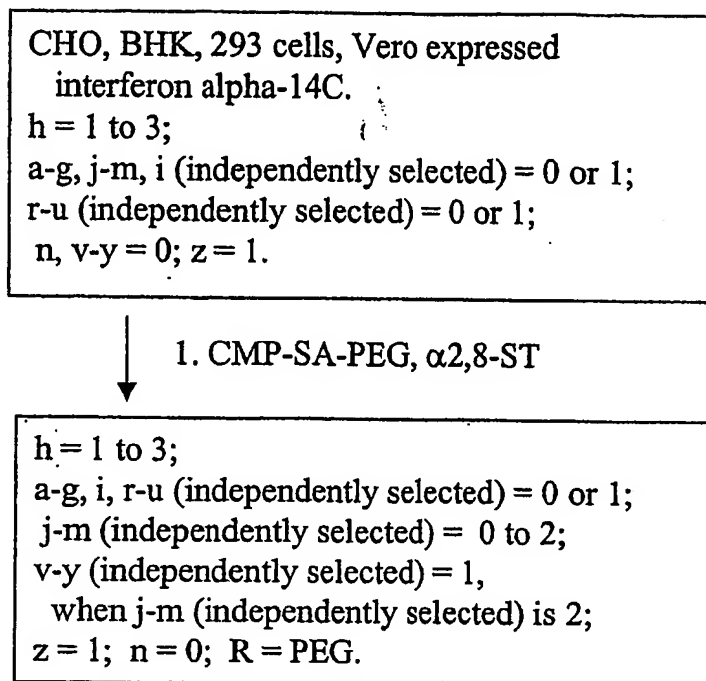


FIG. 30J

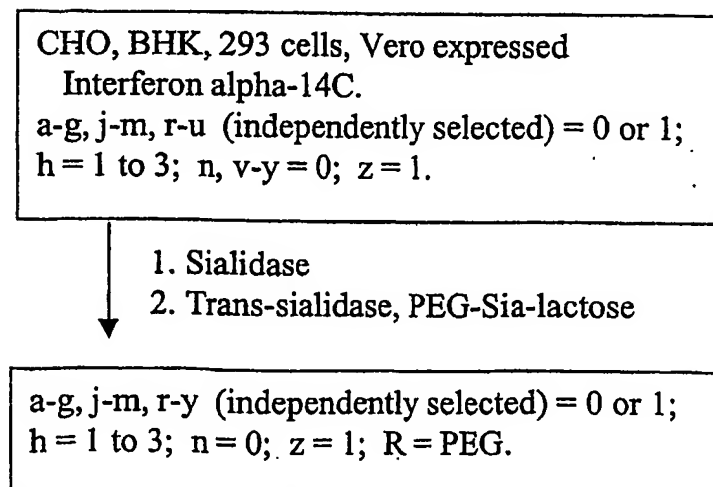


FIG. 30K

71/498

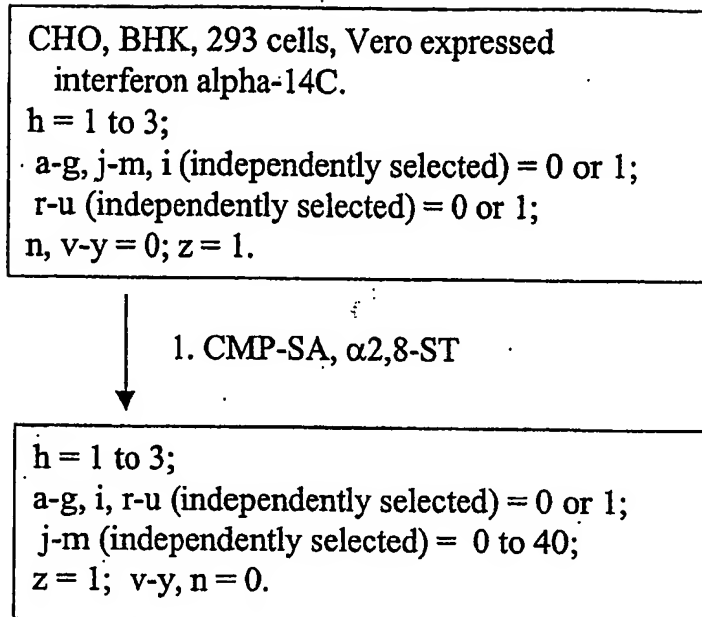


FIG. 30L

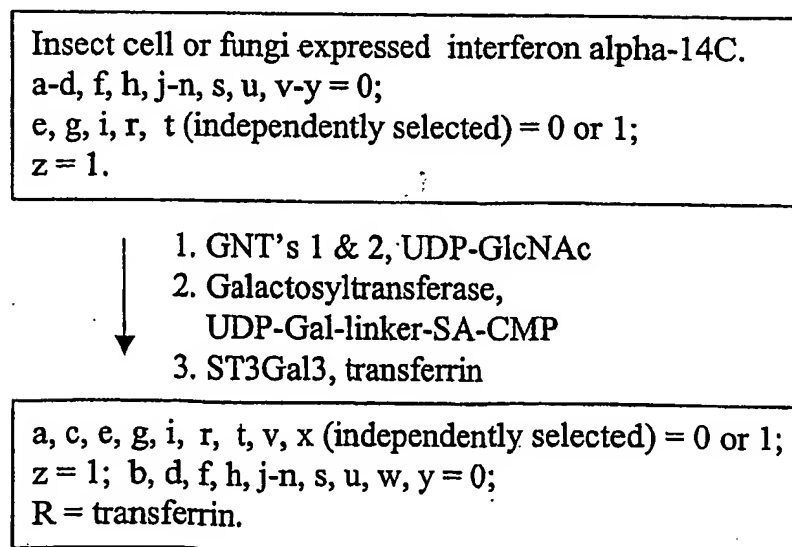


FIG. 30M

72/498

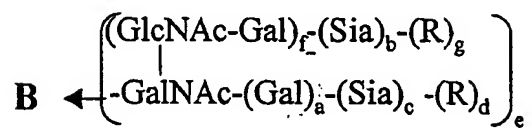
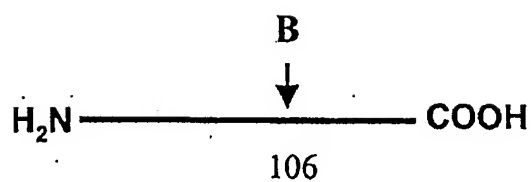
Insect cell or fungi expressed interferon alpha-14C.  
a-d, f, h, j-n, s, u, v-y = 0;  
e, g, i, r, t (independently selected) = 0 or 1; z = 1.

- ↓
1. endoglycanase
  2. Galactosyltransferase,  
UDP-Gal-linker-SA-CMP
  3. ST3Gal3, transferrin

i (independently selected) = 0 or 1;  
a-h, j-m, r-z = 0;  
n = 1; R' = -Gal-linker-transferrin.

FIG. 30N

73/498



a-c, e, f (independently selected) = 0 or 1;  
 d, g = 0; R = polymer, glycoconjugate.

FIG. 300



74/498

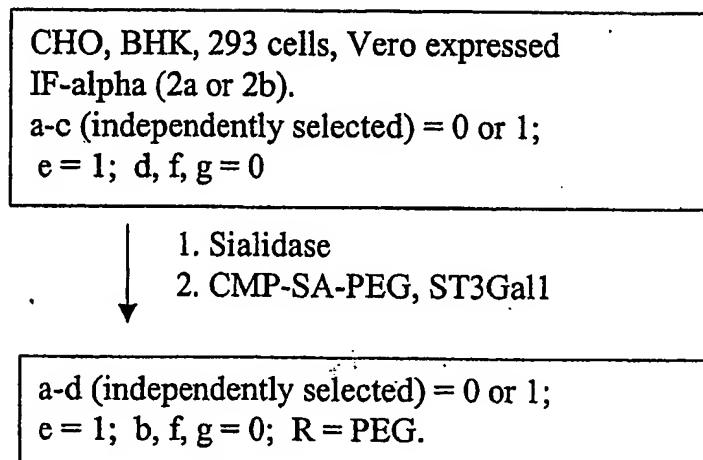


FIG. 30P

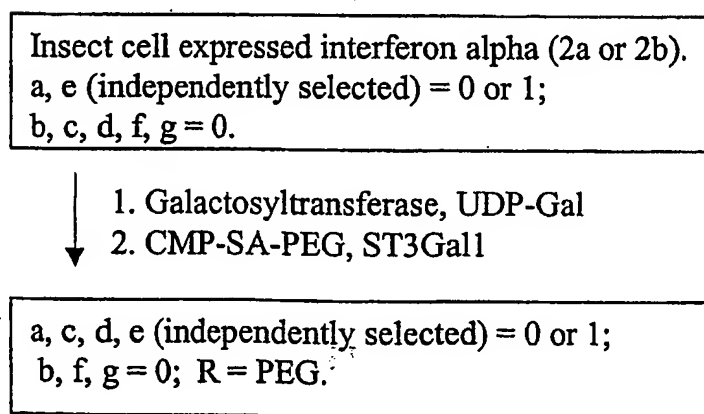


FIG. 30Q

75/498

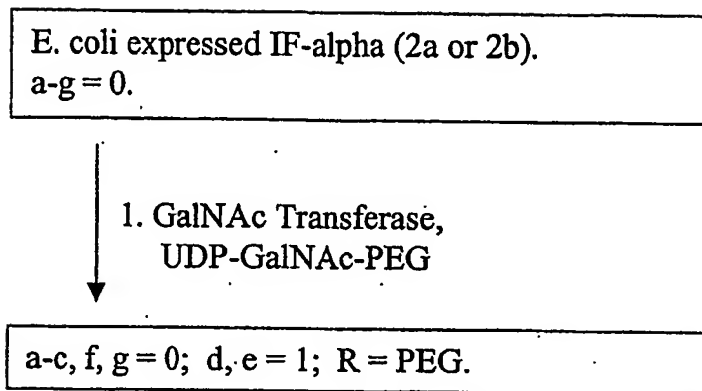


FIG. 30R

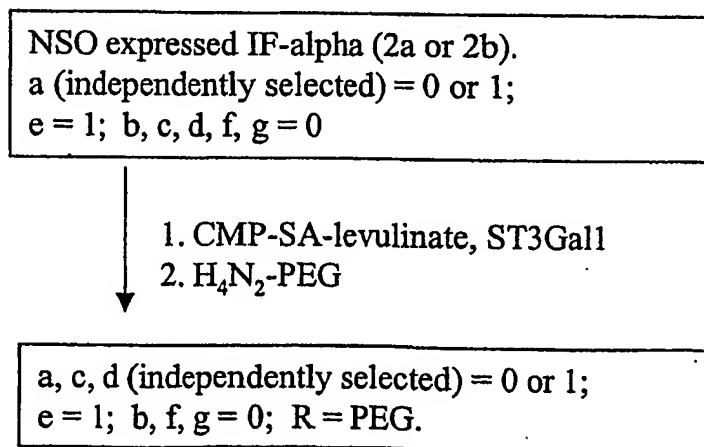


FIG. 30S

76/498

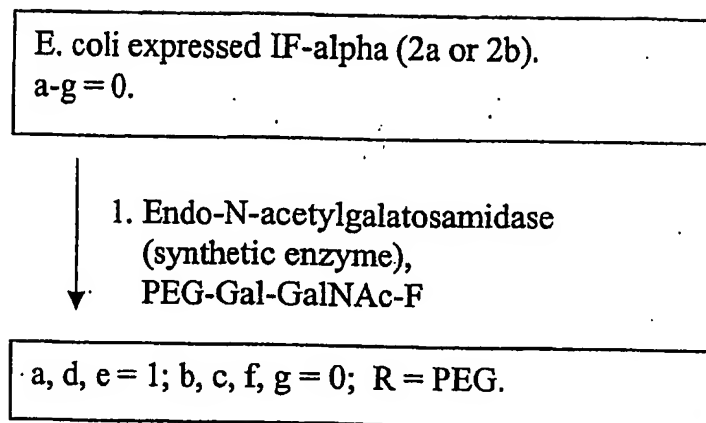


FIG. 30T

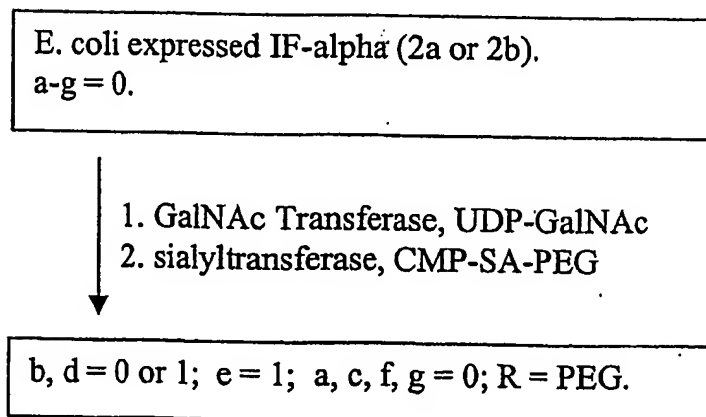


FIG. 30U

77/498

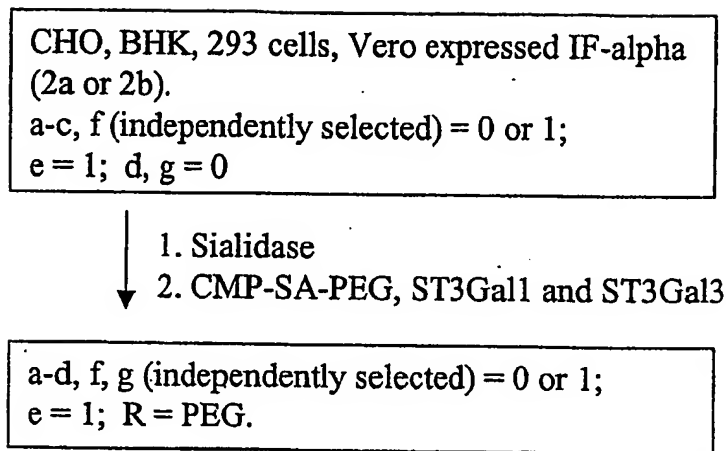


FIG. 30V

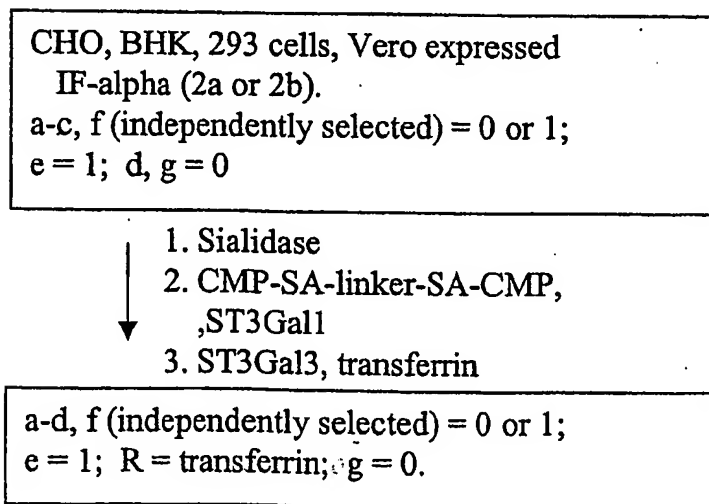
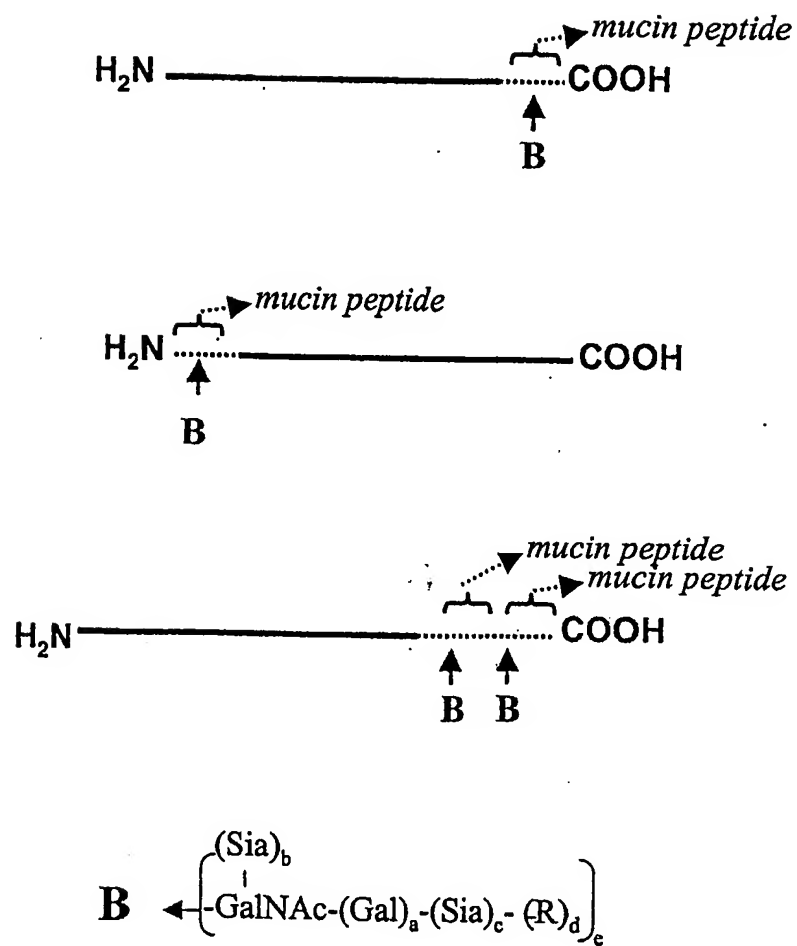


FIG. 30W

78/498



a-c, e (independently selected) = 0 or 1;  
 d = 0; R = polymer, glycoconjugate.

FIG. 30X

79/498

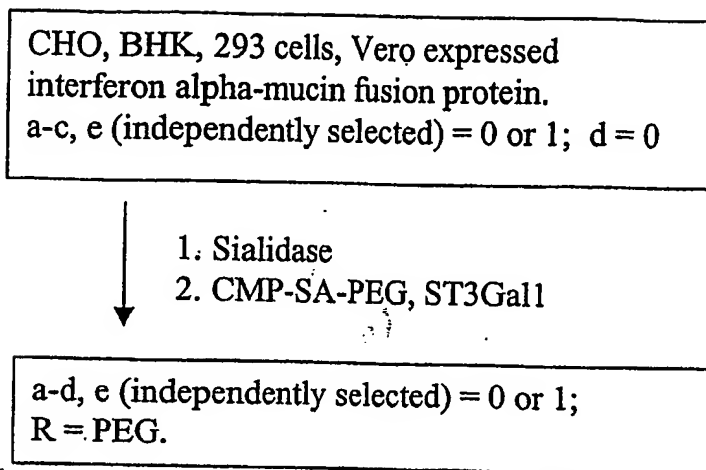


FIG. 30Y

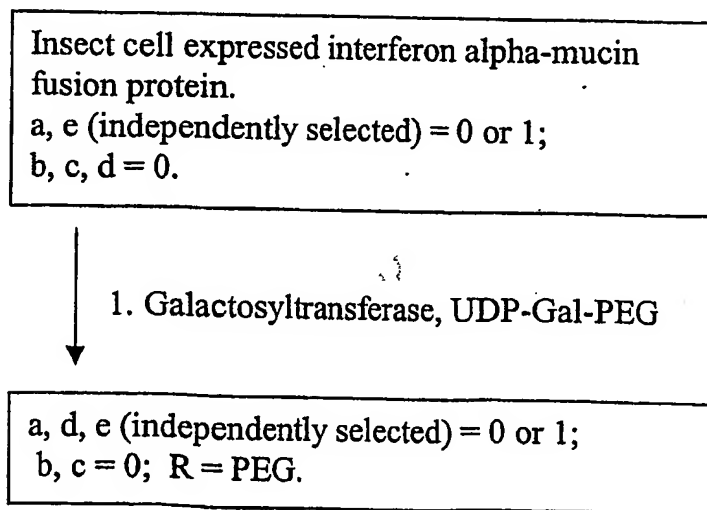


FIG. 30Z

80/498

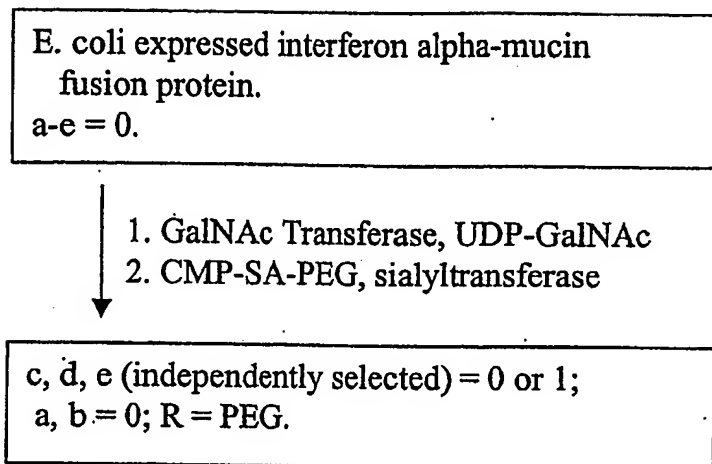
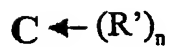
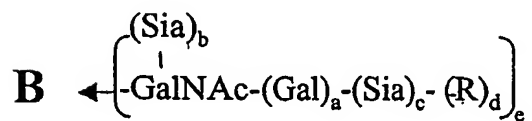
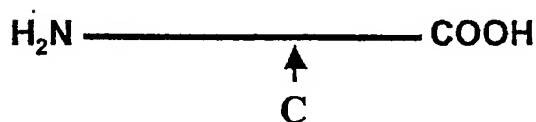
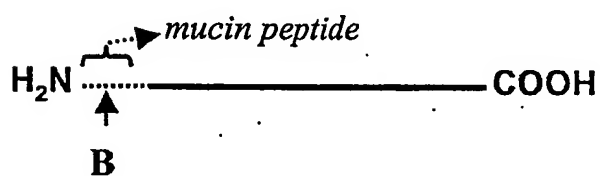
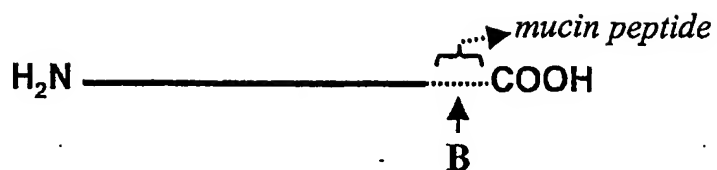


FIG. 30AA

81/498



a-c, e (independently selected) = 0 or 1;  
d = 0; R = polymer, linker.

FIG. 30BB



82/498

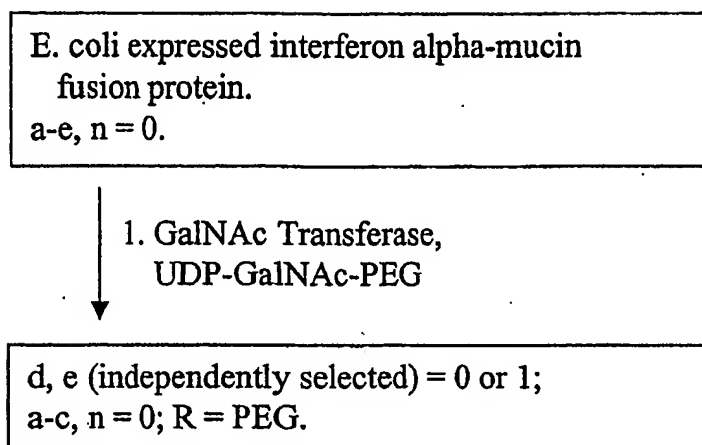


FIG. 30CC

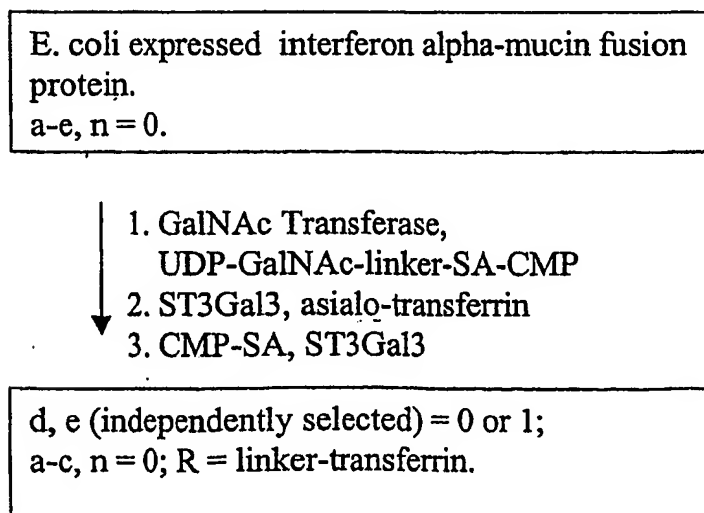


FIG. 30DD

83/498

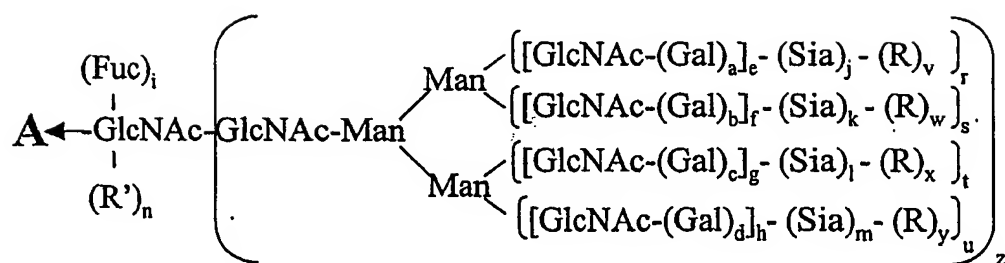
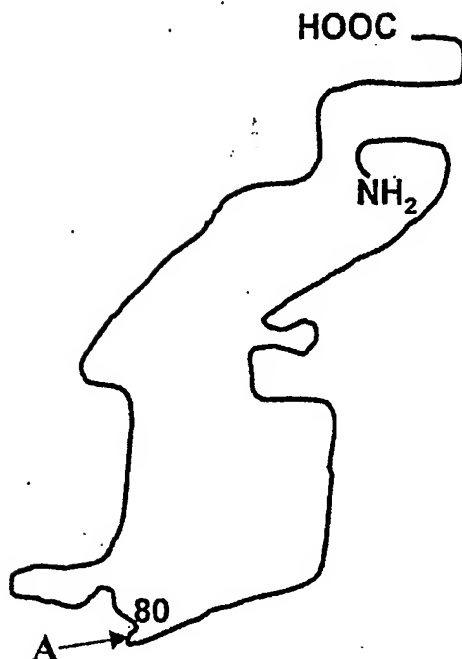
E. coli expressed Interferon alpha (no fusion).  
a-e, n = 0.

- ↓  
1. NHS-CO-linker-SA-CMP  
2. ST3Gal3, transferrin

a-e = 0; n = 1; R' = linker-transferrin.

FIG. 30EE

84/498



a-d, i, r-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 or 1.

n, v-y = 0; z = 0 or 1; R = polymer

FIG. 31A

85/498

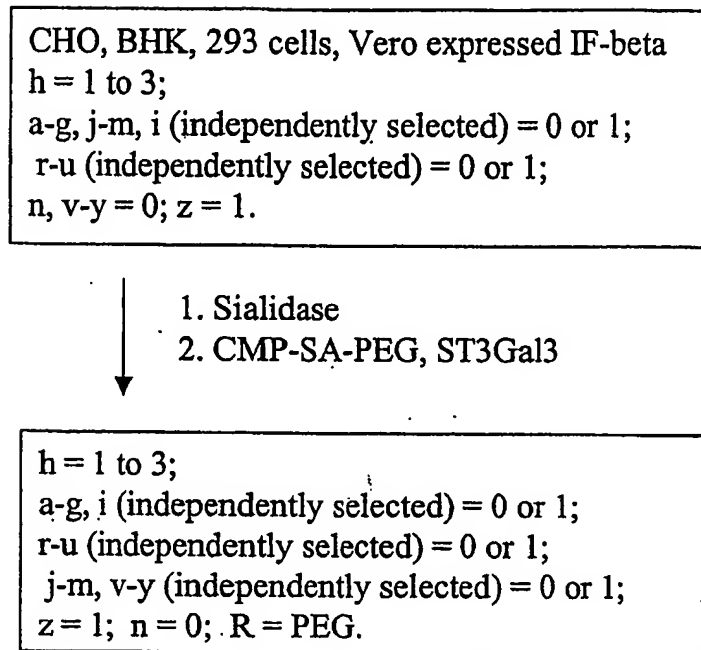


FIG. 31B

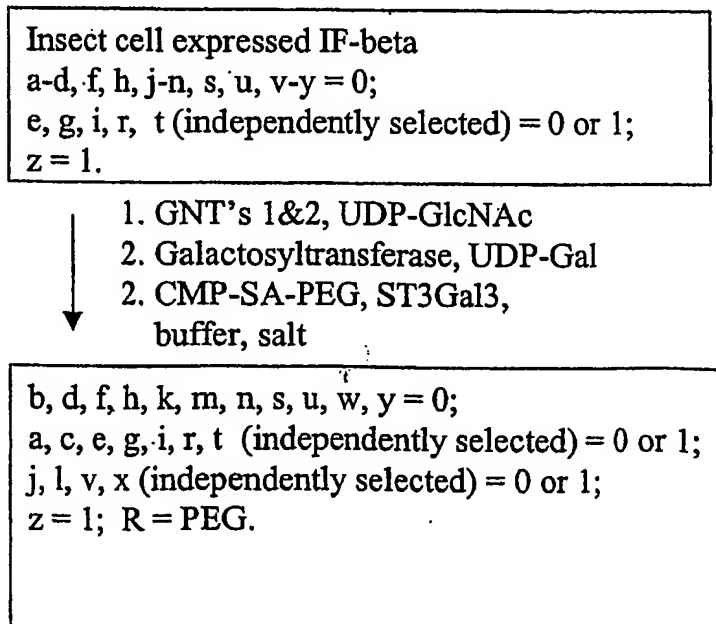


FIG. 31C

86/498

Yeast expressed IF-beta  
 $a-n = 0; z = 1;$   
 $r-y$  (independently selected) = 0 to 1;  
 $R$  (branched or linear) = Man, oligomannose or polysaccharide.

- ↓ 1. Endo-H  
 2. Galactosyltransferase, UDP-Gal  
 3. CMP-SA-PEG, ST3Gal3

$a-m, r-z = 0; n = 1; R' = -Gal-Sia-PEG.$

FIG. 31D

CHO, BHK, 293 cells, Vero expressed IF-beta  
 $h = 1$  to 3;  
 $a-g, j-m, i$  (independently selected) = 0 or 1;  
 $r-u$  (independently selected) = 0 or 1;  
 $n, v-y = 0; z = 1.$

- ↓ 1. CMP-SA-PEG, ST3Gal3

$h = 1$  to 3;  
 $a-g, i$  (independently selected) = 0 or 1;  
 $r-u$  (independently selected) = 0 or 1;  
 $j-m, v-y$  (independently selected) = 0 or 1;  
 $z = 1; n = 0; R = PEG.$

FIG. 31E

87/498

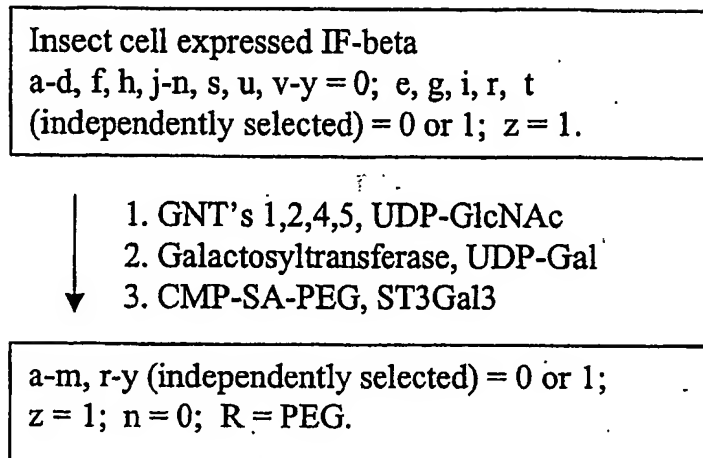


FIG. 31F

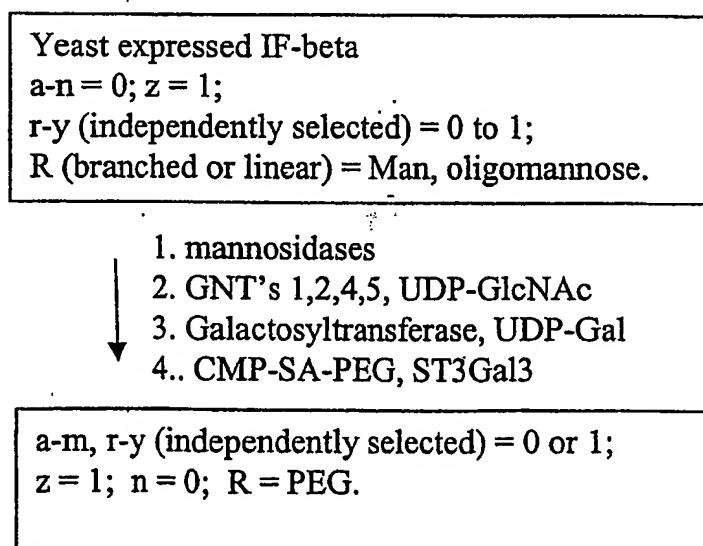


FIG. 31G

88/498

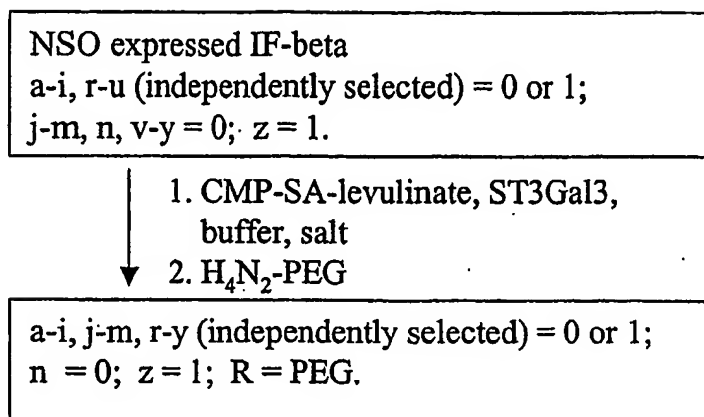


FIG. 31H

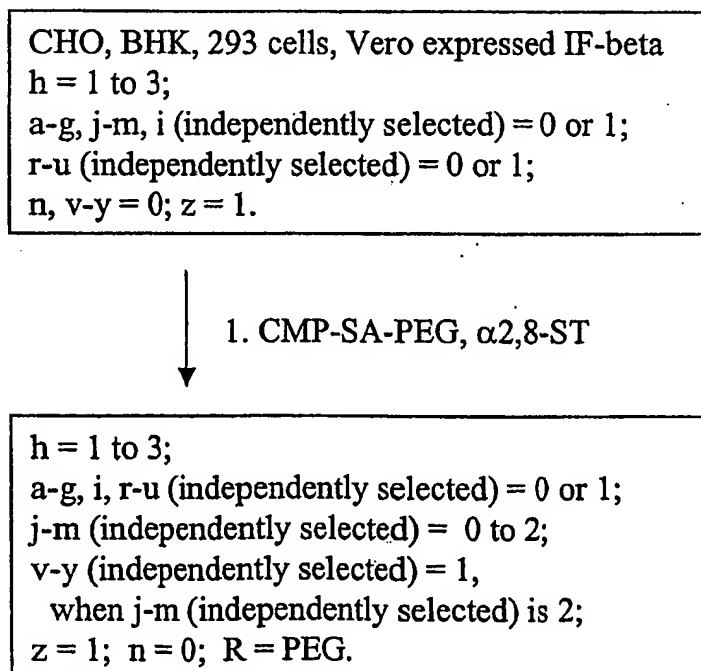


FIG. 31I

89/498

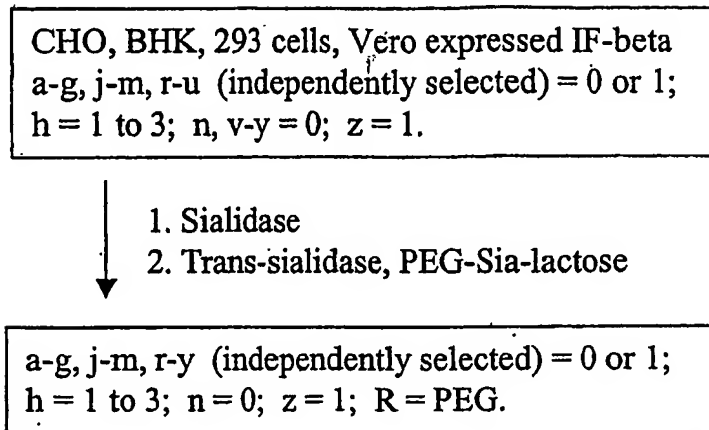


FIG. 31J

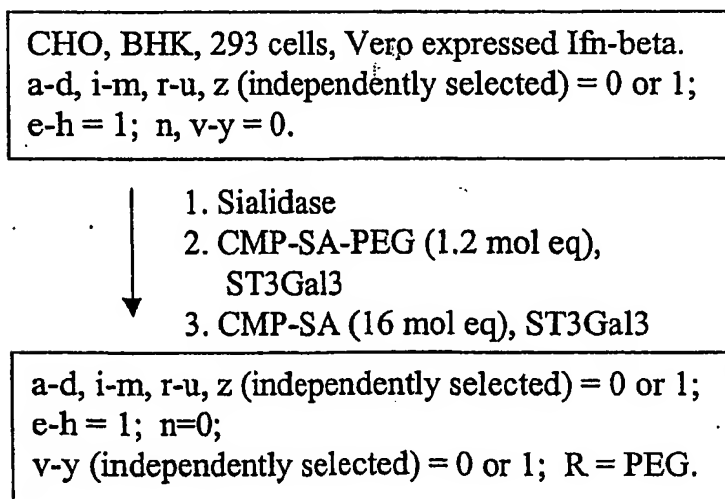


FIG. 31K



90/498

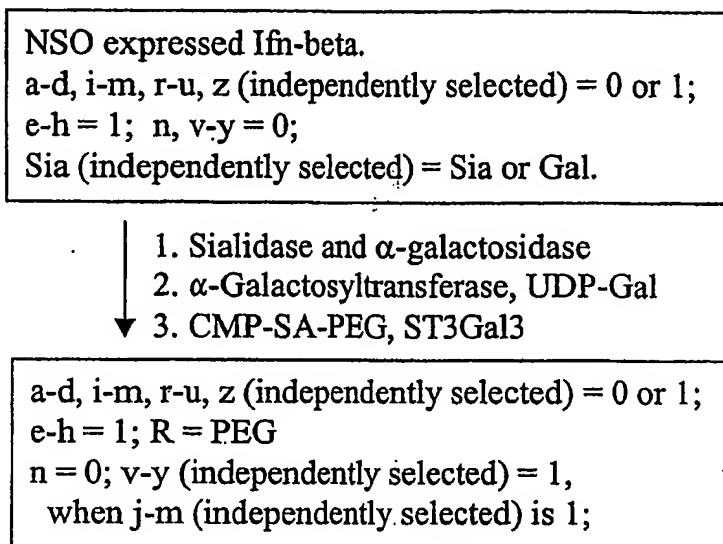


FIG. 31L

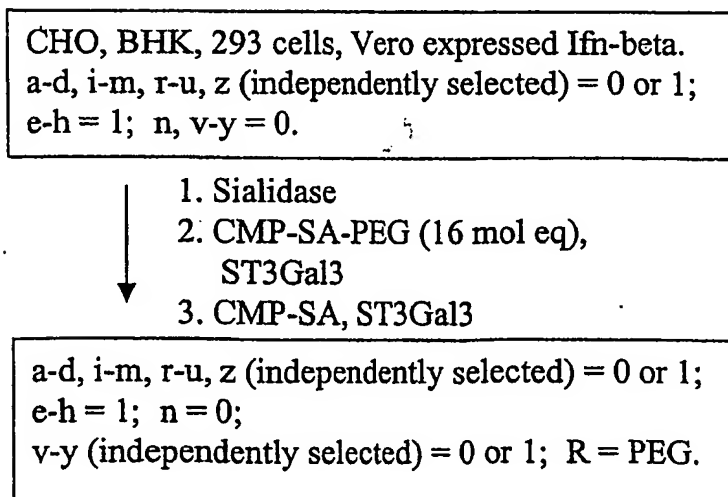


FIG. 31M

91/498

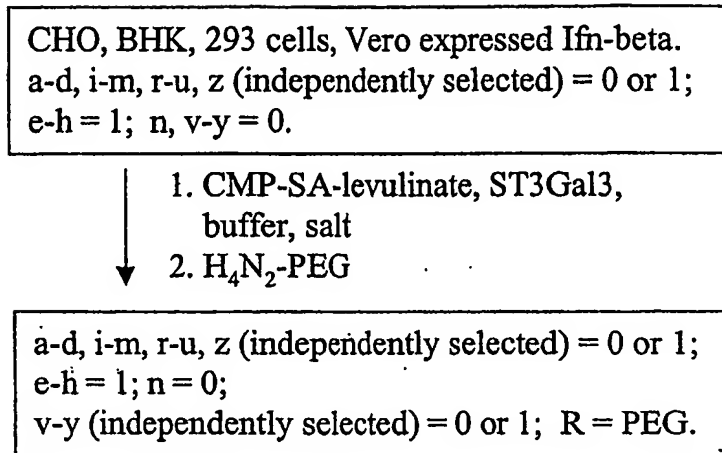


FIG. 31N

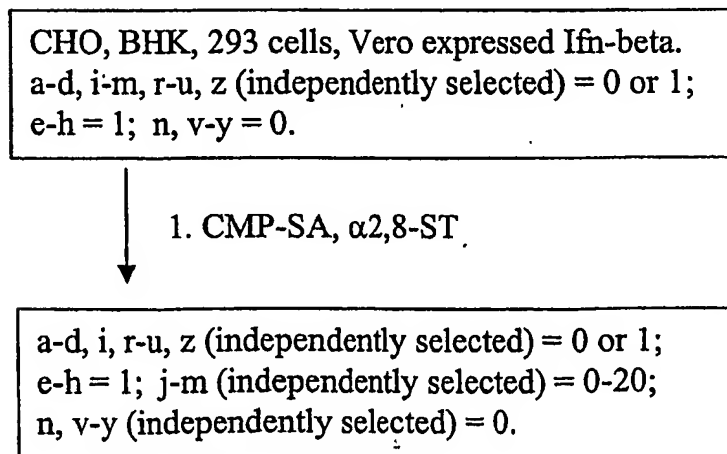
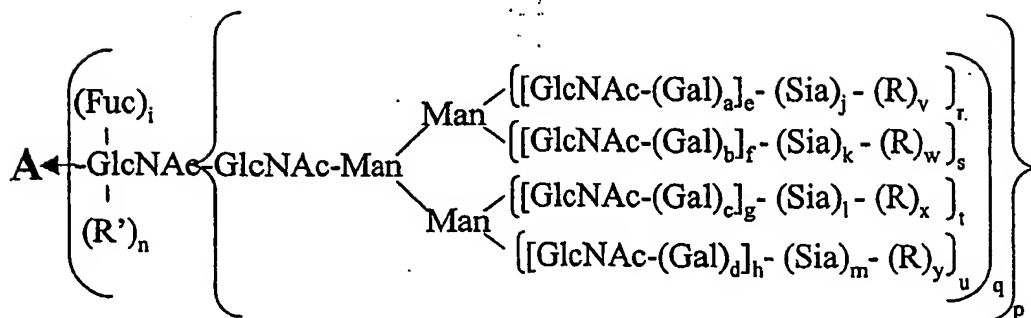
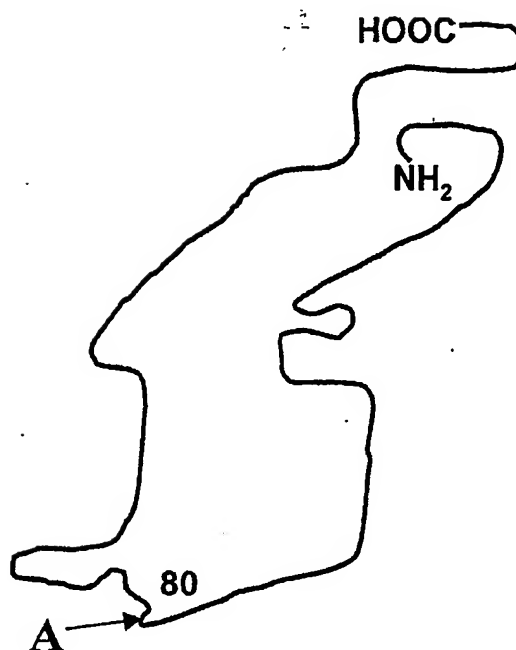


FIG. 31O

92/498



a-d, i, p-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = modifying group;

R' = H, glycosyl group, modifying group,  
glycoconjugate.

FIG. 31P

93/498

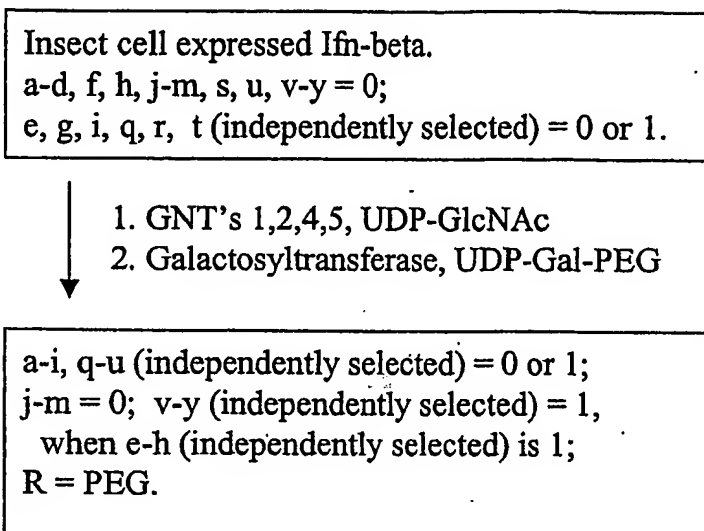


FIG. 31Q

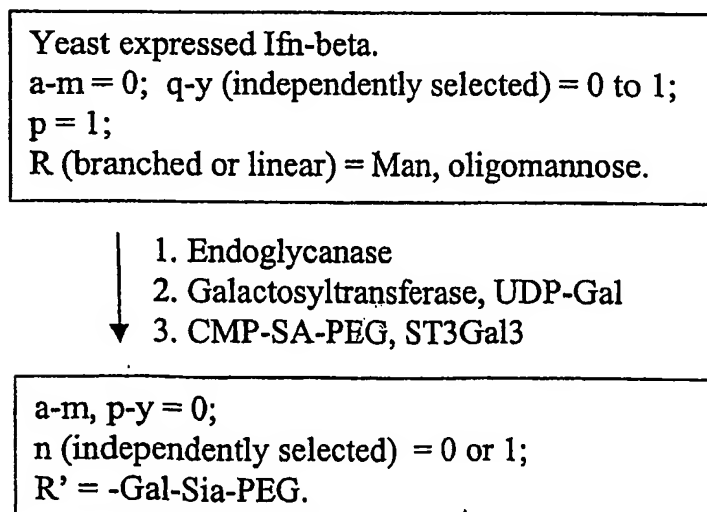


FIG. 31R

94/498

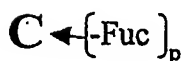
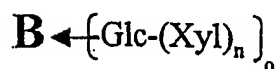
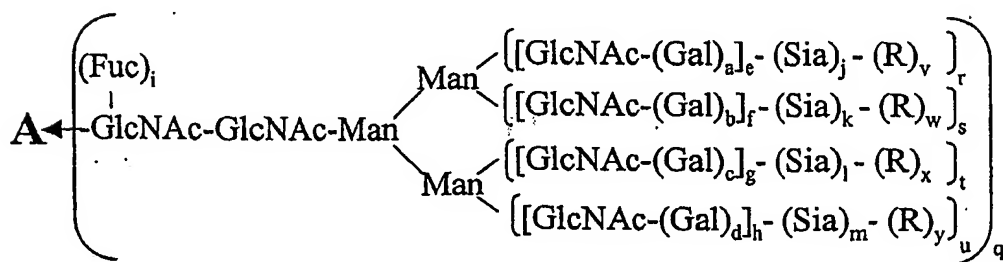
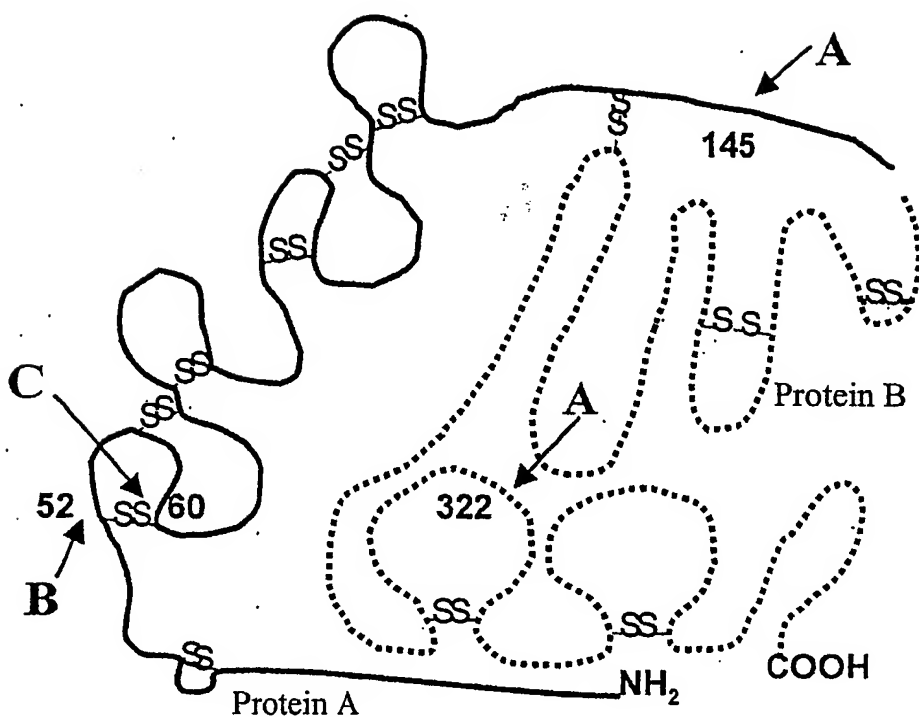
CHO, BHK, 293 cells, Vero expressed Ifn-beta.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. CMP-SA-linker-SA-CMP,  
ST3Gal3
  2. ST3Gal3, desialylated transferrin.
  3. CMP-SA, ST3Gal3

a-m, q-u (independently selected) = 0 or 1;  
p = 1; n = 0;  
v-y (independently selected) = 0 or 1;  
R = linker-transferrin.

FIG. 31S

95/498



a-d, i, q-u (independently selected) = 0 or 1.

o, p (independently selected) = 0 or 1.

e-h, n (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 20.

v-y = 0;

R = modifying group, mannose, oligo-mannose, Sia-Lewis X, Sia-Lewis A..

FIG. 32A

96/498

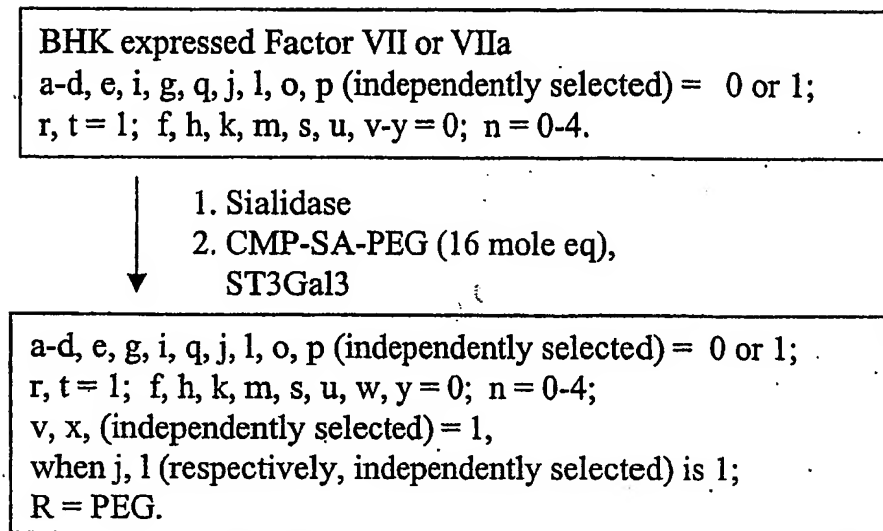


FIG. 32B

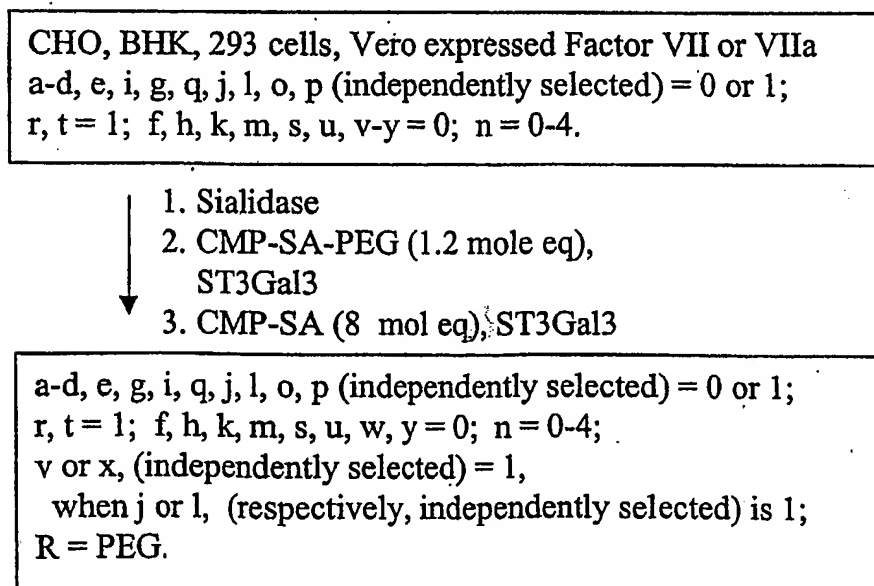


FIG. 32C

97/498

NSO expressed Factor VII or VIIa  
a--u (independently selected) = 0 or 1;  
v-y = 0; n = 0-4;  
Sia (independently selected) = Sia or Gal.

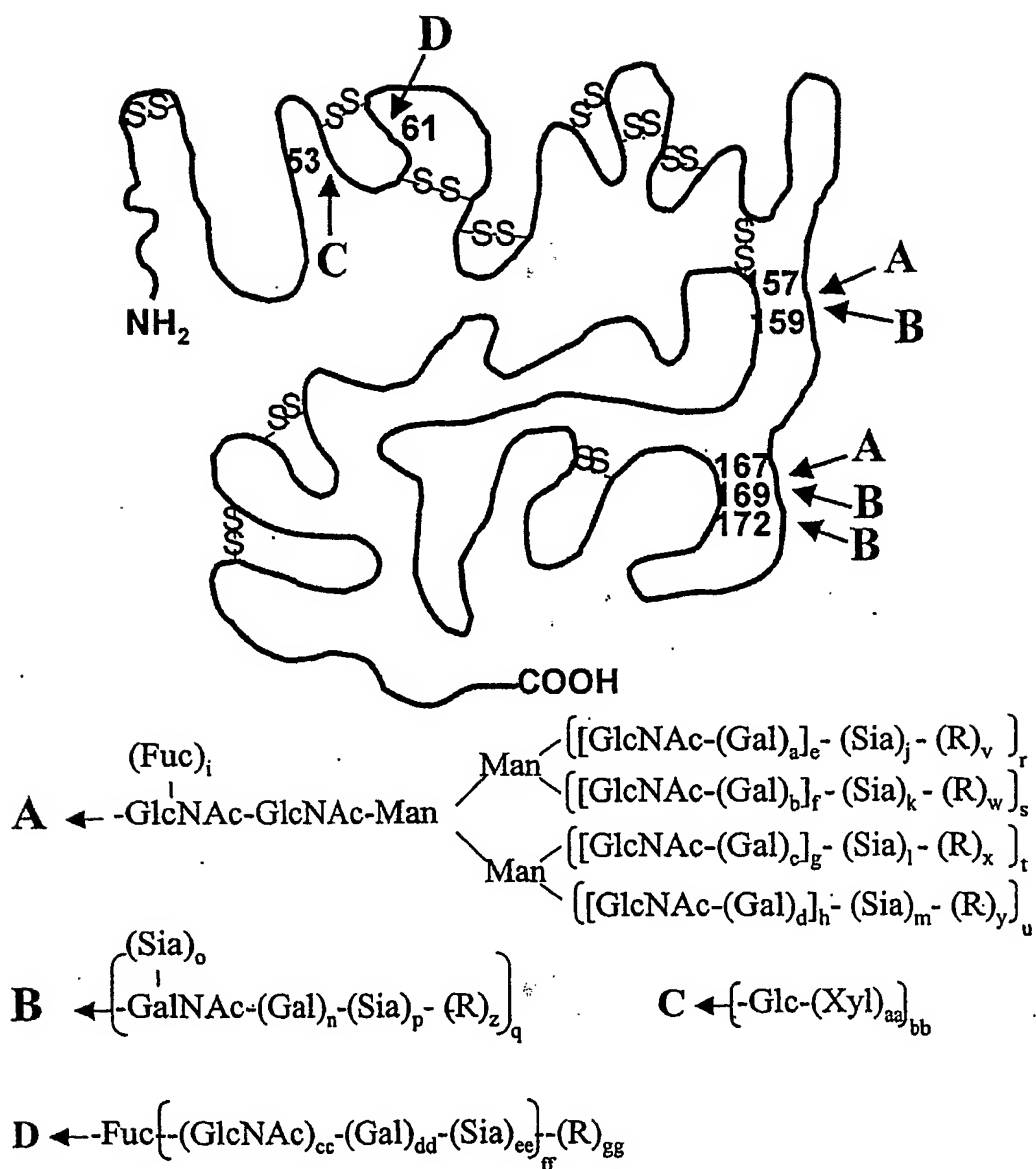
- ↓  
1. Sialidase and  $\alpha$ -galactosidase  
2. Galactosyltransferase, UDP-Gal  
↓ 3. CMP-SA-PEG, ST3Gal3

a-m, o-u (independently selected) = 0 or 1;  
n = 0-4; v-y (independently selected) = 1,  
when j-m (independently selected) is 1;  
Sia = Sia; R = PEG.

FIG. 32D



98/498



a-d, i, n-u (independently selected) = 0 or 1.

bb, cc, dd, ee, ff, gg (independently selected) = 0 or 1.

e-h, aa (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 20.

v-z = 0; R = modifying group, mannose, oligo-mannose.

FIG. 33A

99/498

CHO, BHK, 293 cells, Vero expressed Factor IX  
 a-d, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, j-m, i, n, o, p, r-u (independently  
 selected) = 0 or 1;  
 v-z, gg = 0.



1. Sialidase
2. CMP-SA-PEG, ST3Gal3

a-d, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, i, n, r-u (independently selected)  
 = 0 or 1;  
 o, p, z = 0;  
 j-m, ee, v-y, gg (independently selected) = 0 or 1;  
 R = PEG.

FIG. 33B

CHO, BHK, 293 cells, Vero expressed Factor IX  
 a-d, n, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, j-m, i, o, p, r-u (independently  
 selected) = 0 or 1;  
 v-z, gg = 0.



1. Sialidase
2. CMP-SA-PEG, ST3Gal3
3. ST3Gal1, CMP-SA

a-d, n, p, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, i, r-u (independently selected) =  
 0 or 1;  
 j-m, ee, v-y, gg (independently selected) = 0 or 1;  
 o, z = 0; R = PEG.

FIG. 33C

100/498

CHO, BHK, 293 cells, Vero expressed Factor IX  
 a-d, n, q, bb, cc, dd, ff = 1; e-h, aa = 1 to 4; ee, j-m, i,  
 o, p, r-u (independently selected) = 0 or 1; v-z, gg = 0.

1. sialidase
2. Galactosyltransferase, UDP-Gal
3. CMP-SA, ST3Gal3
4. CMP-SA-PEG, ST3Gal1

a-d, n, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, i, r-u (independently selected) =  
 0 or 1; R = PEG;  
 o, v-y, gg = 0;  
 j-m, p, ee (independently selected) = 0 or 1, but when  
 p = 1, z = 1.

FIG. 33D

CHO, BHK, 293 cells, Vero expressed Factor IX  
 a-d, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, j-m, i, n, o, p, r-u (independently  
 selected) = 0 or 1;  
 v-z, gg = 0.

CMP-SA-PEG, ST3Gal3

a-d, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, i, n, r-u (independently selected)  
 = 0 or 1; R = PEG;  
 o, p, z = 0; j-m, ee, v-y, gg (independently selected) =  
 0 or 1.

FIG. 33E

101/498

CHO, BHK, 293 cells, Vero expressed Factor IX  
 a-d, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, j-m, i, n, o, p, r-u (independently  
 selected) = 0 or 1;  
 v-z, gg = 0.

- ↓
1. CMP-SA-levulinate, ST3Gal3,  
buffer, salt
  2. H<sub>4</sub>N<sub>2</sub>-PEG

a-d, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, i, n, r-u (independently selected)  
 = 0 or 1;  
 o, p, z = 0; R = PEG;  
 j-m, ee, v-y, gg (independently selected) = 0 or 1.

FIG. 33F

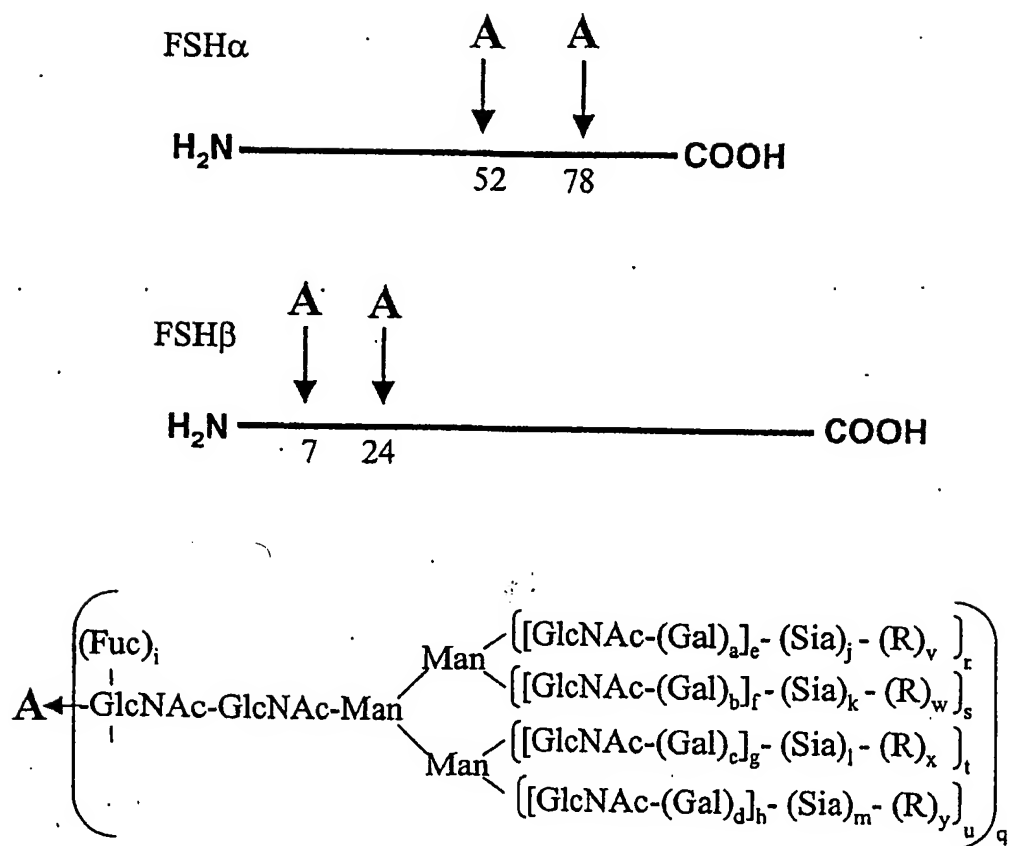
CHO, BHK, 293 cells, Vero expressed Factor IX  
 a-d, n, q, bb, cc, dd, ff = 1;  
 e-h, aa = 1 to 4;  
 ee, j-m, i, o, p, r-u (independently selected) = 0 or 1;  
 v-z, gg = 0.

- ↓
1. CMP-SA-PEG,  $\alpha$ 2,8-ST

a-d, q = 1; e-h = 1 to 4;  
 aa, bb, cc, dd, ee, ff, i, n, r-u (independently selected)  
 = 0 or 1;  
 o, p, z = 0; R = PEG;  
 j-m, ee (independently selected) = 0 to 2;  
 v-y, gg (independently selected) = 1, when j-m  
 (independently selected) is 2;

FIG. 33G

102/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0;

R = modifying group, mannose, oligo-mannose.

FIG. 34A

103/498

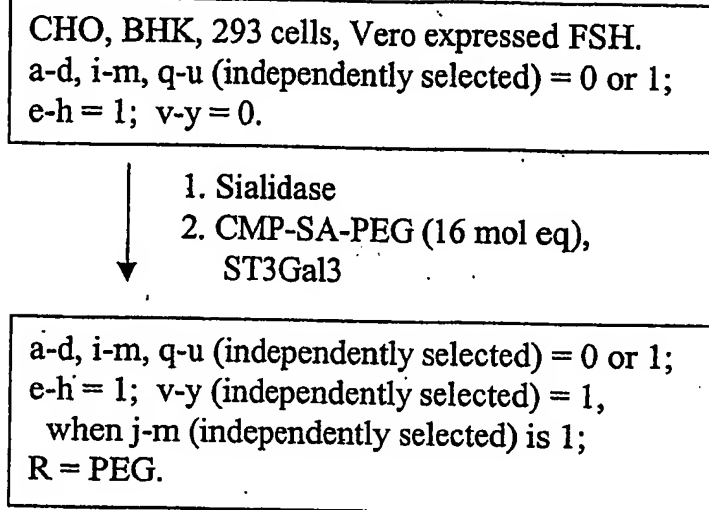


FIG. 34B

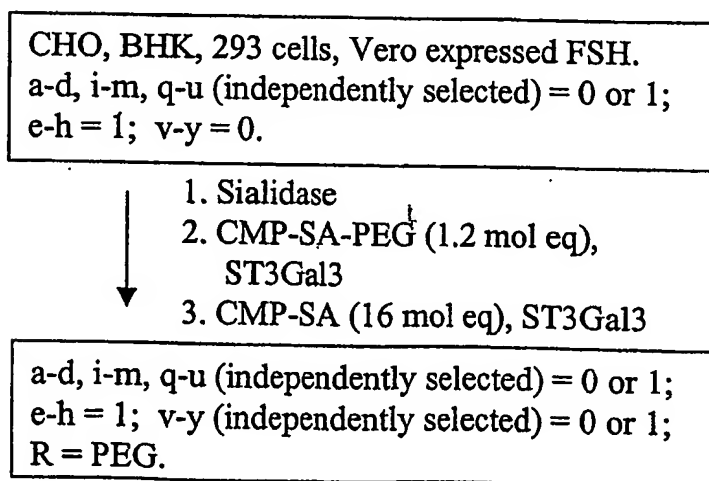


FIG. 34C

104/498

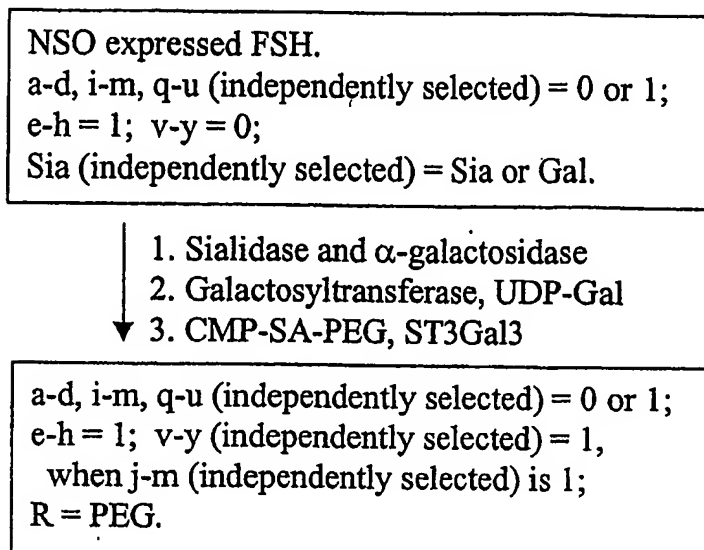


FIG. 34D

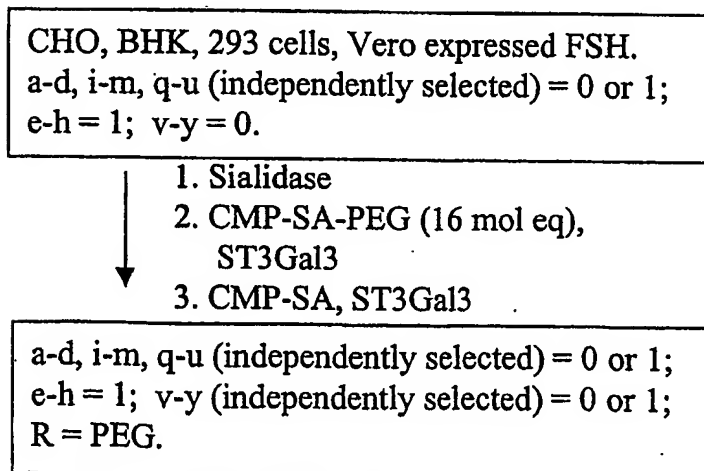


FIG. 34E

105/498

CHO, BHK, 293 cells, Vero expressed FSH.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. CMP-SA-levulinate, ST3Gal3,  
buffer, salt
  2. H<sub>4</sub>N<sub>2</sub>-PEG

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y (independently selected) = 0 or 1;  
R = PEG.

FIG. 34F

CHO, BHK, 293 cells, Vero expressed FSH.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. CMP-SA,  $\alpha$ 2,8-ST

a-d, i, q-u (independently selected) = 0 or 1;  
e-h = 1; j-m (independently selected) = 0-20;  
v-y (independently selected) = 0.

FIG. 34G



106/498

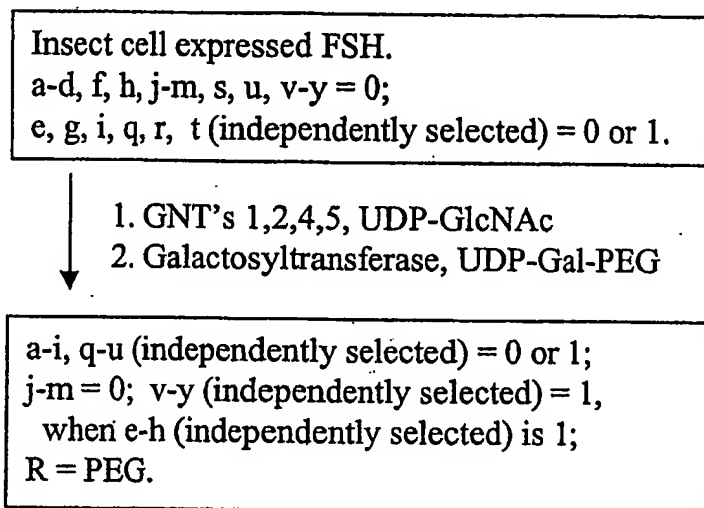


FIG. 34H

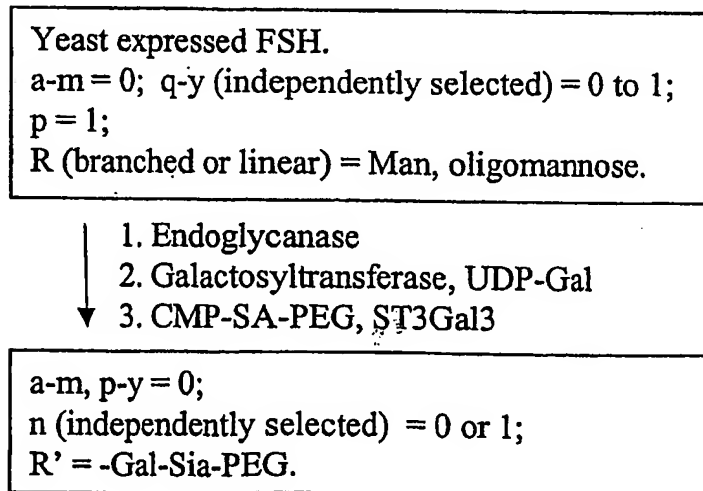


FIG. 34I

107/498

CHO, BHK, 293 cells, Vero expressed FSH.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. CMP-SA-linker-SA-CMP, ST3Gal3
  2. ST3Gal1, desialylated chorionic gonadrophin (CG) produced in CHO.
  3. CMP-SA, ST3Gal3, ST3Gal1

a-m, q-u (independently selected) = 0 or 1;  
p = 1; n = 0;  
v-y (independently selected) = 0 or 1;  
R = linker-CG.

FIG. 34J

108/498

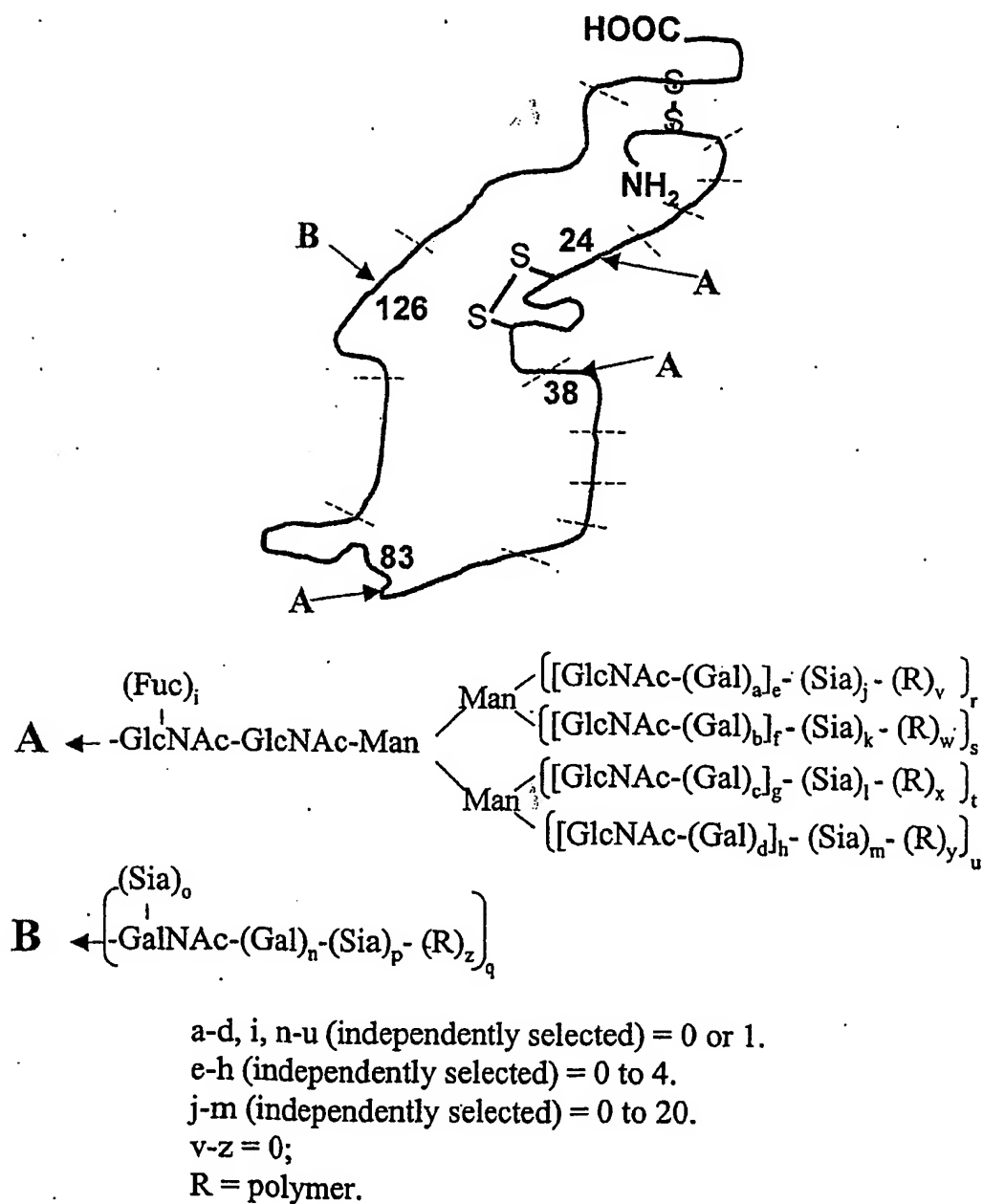


FIG. 35A

109/498

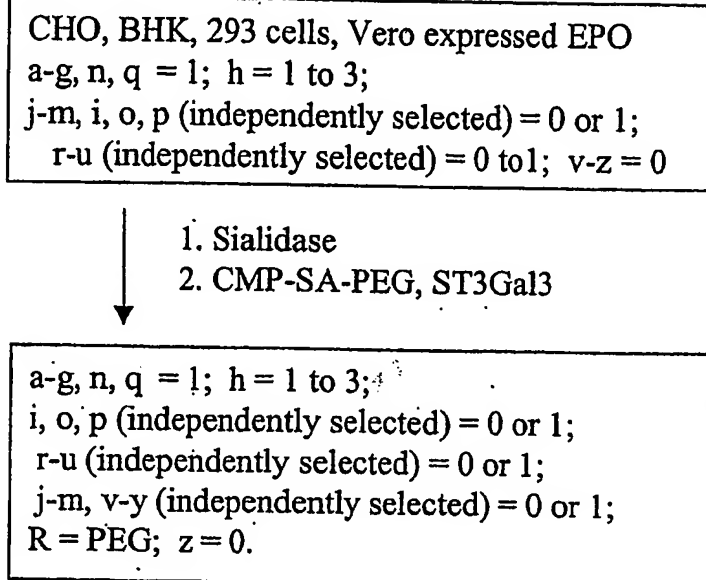


FIG. 35B

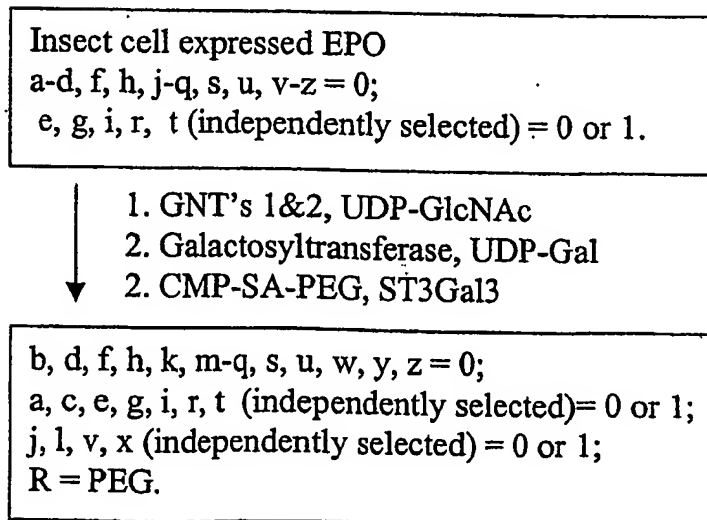


FIG. 35C

110/498

CHO, BHK, 293 cells, Vero expressed EPO  
 a-q, r-u (independently selected) = 0 or 1;  
 v-z = 0.

1. sialidase
2. Galactosyltransferase, UDP-Gal
3. CMP-SA, ST3Gal3
4. CMP-SA-PEG, ST3Gal1

a-h, n, q = 1;  
 i-m, o, r-u (independently selected) = 0 or 1;  
 v-y = 0; p, z = 0 or 1; R = PEG.

FIG. 35D

CHO, BHK, 293 cells, Vero expressed EPO  
 a-g, n, q = 1; h = 1 to 3;  
 j-m, i, o, p (independently selected) = 0 or 1;  
 r-u (independently selected) = 0 or 1;  
 v-z = 0

1. CMP-SA-PEG, ST3Gal3

a-g, n, q = 1; h = 1 to 3;  
 i, o, p (independently selected) = 0 or 1;  
 r-u (independently selected) = 0 to 1;  
 j-m, v-y (independently selected) = 0 or 1;  
 R = PEG; z = 0.

FIG. 35E

111/498

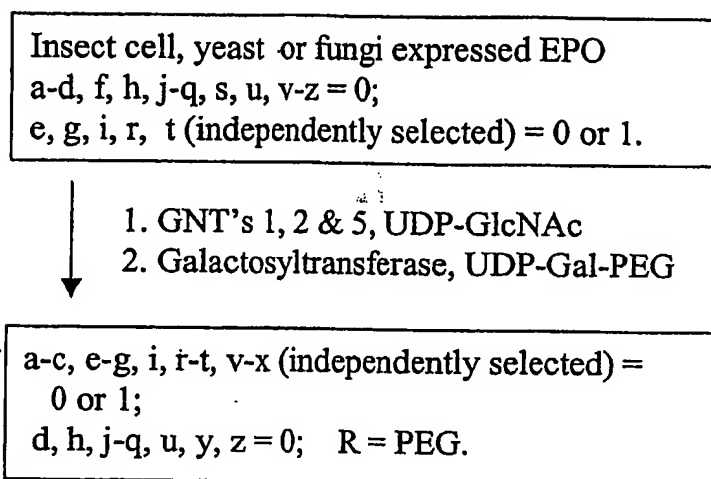


FIG. 35F

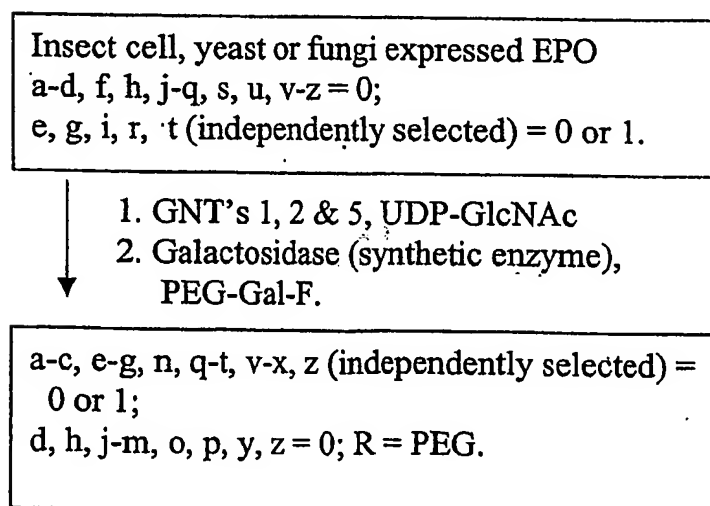


FIG. 35G

112/498

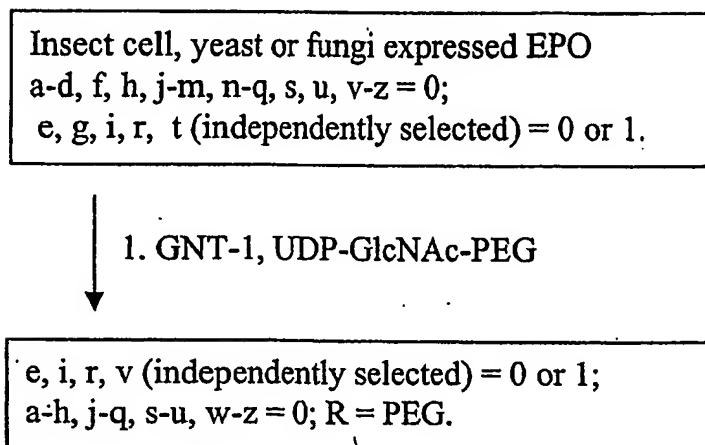


FIG. 35H

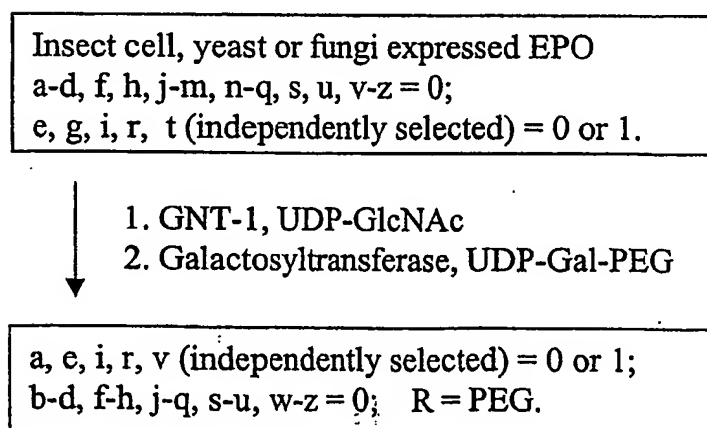


FIG. 35I

113/498

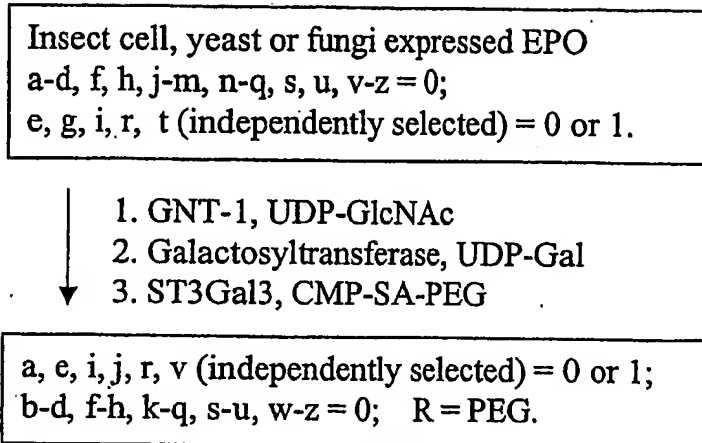


FIG. 35J

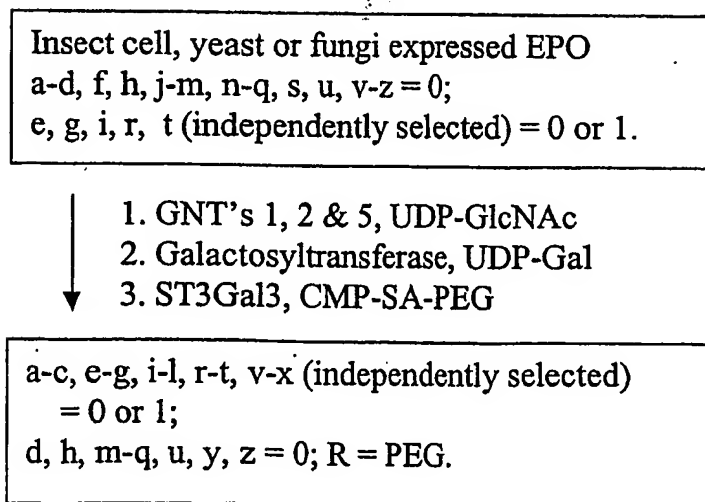


FIG. 35K



114/498

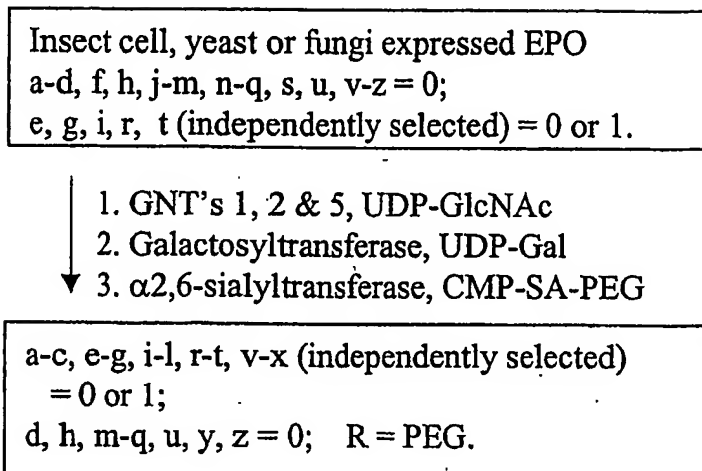


FIG. 35L

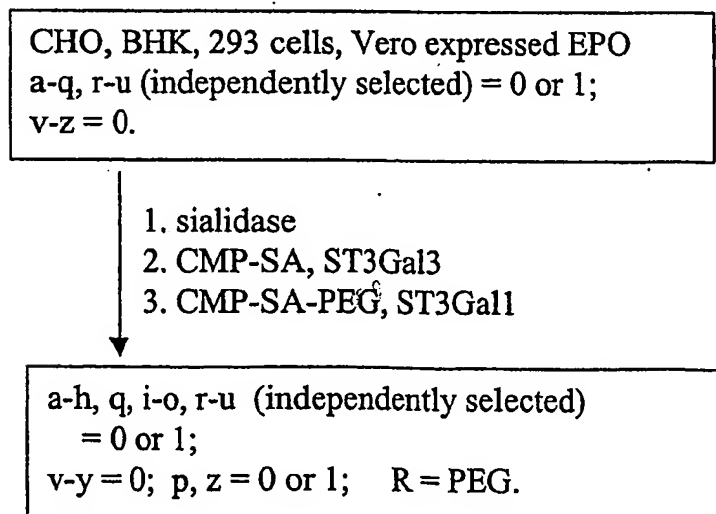


FIG. 35M

115/498

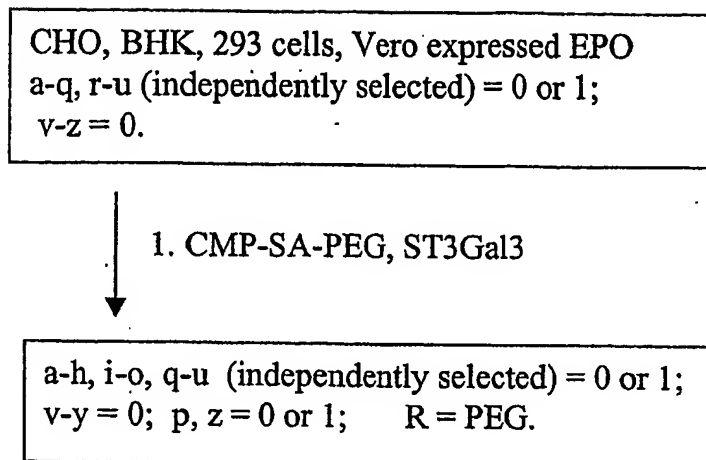


FIG. 35N

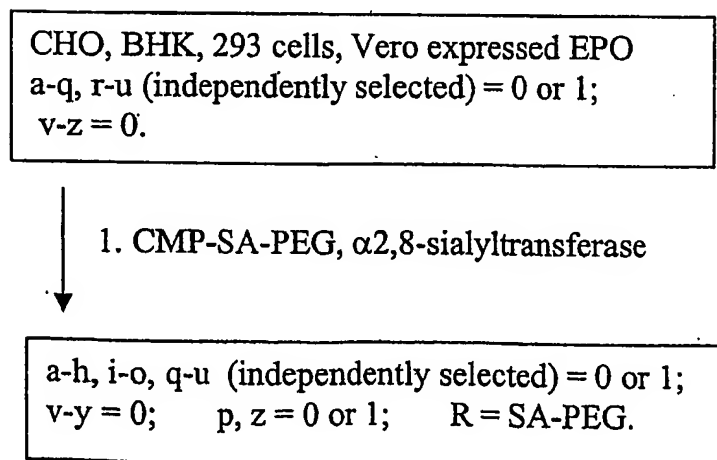


FIG. 35O

116/498

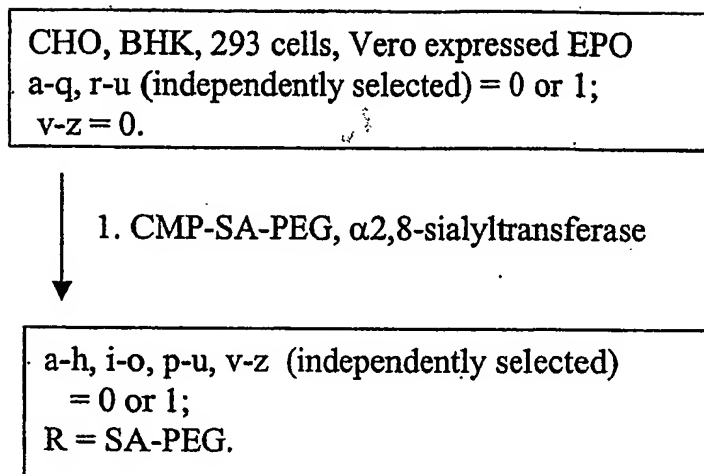


FIG. 35P

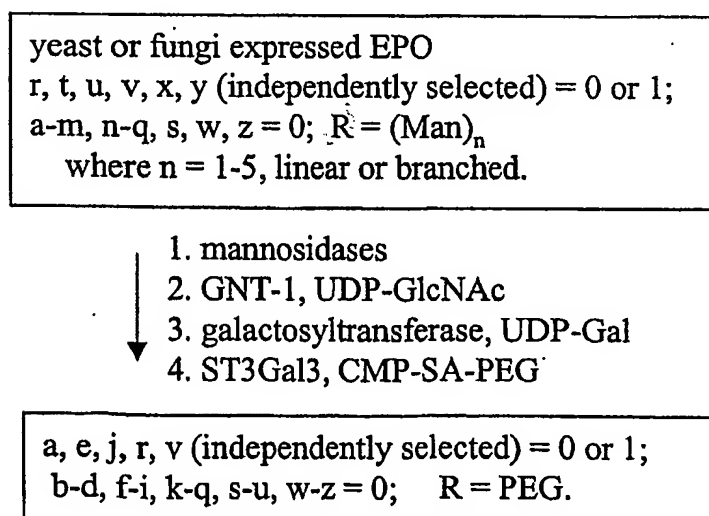


FIG. 35Q

117/498

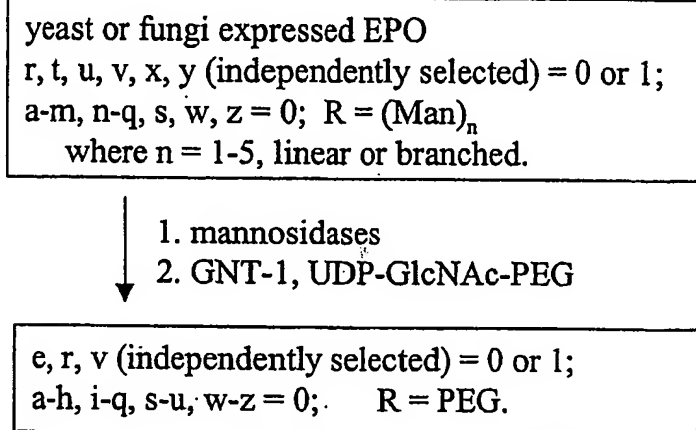


FIG. 35R

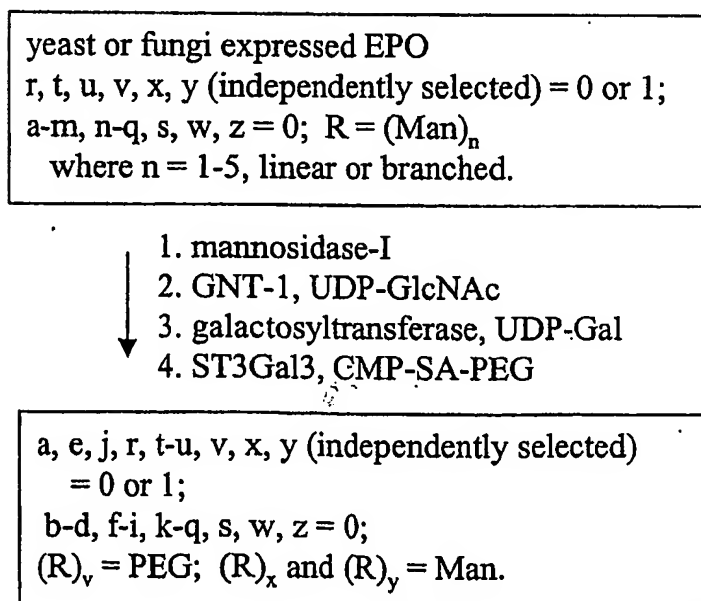
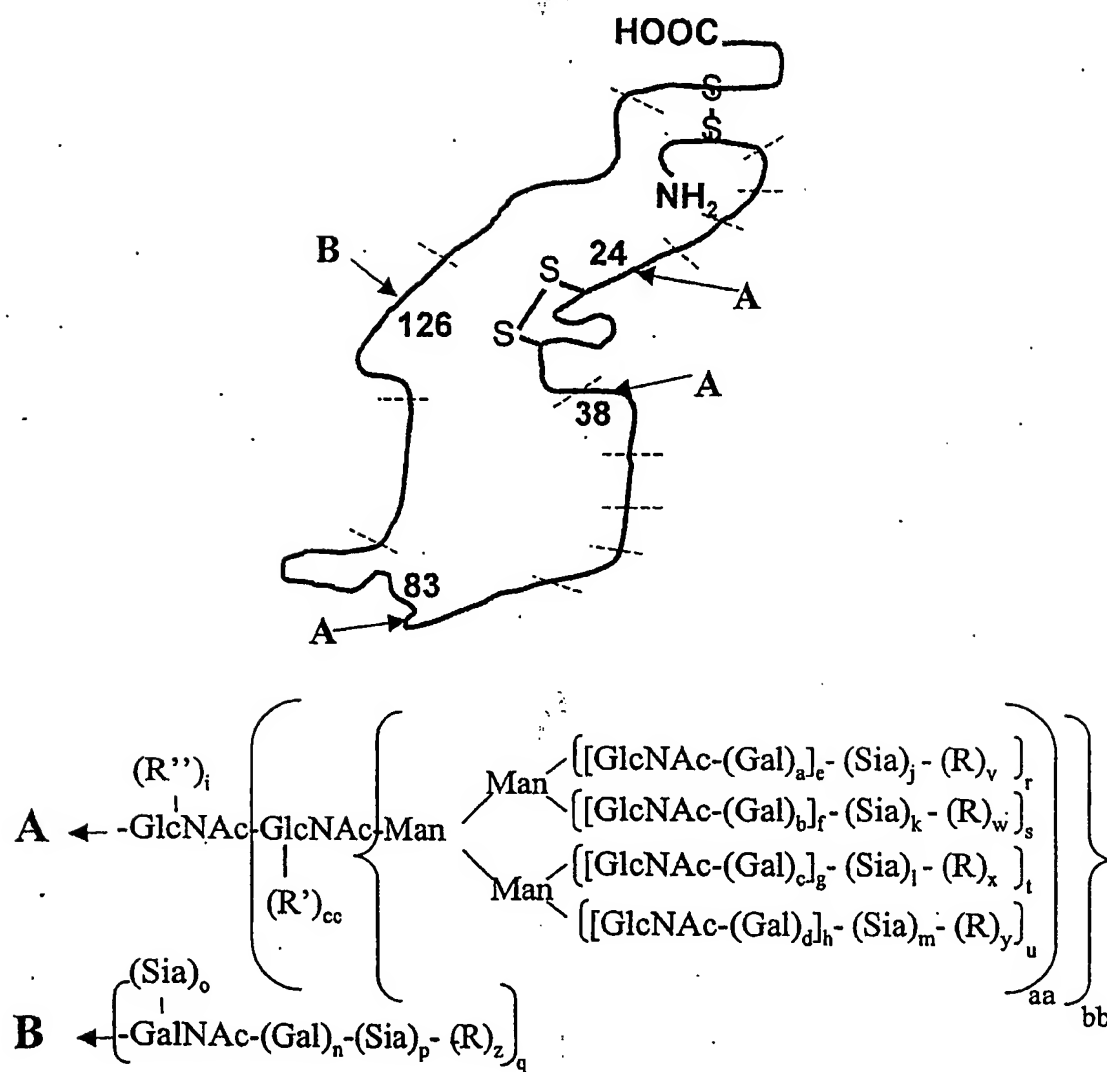


FIG. 35S

118/498



a-d, i, n-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 to 20.

v-z = 0; aa, bb = 1; cc = 0;

R = polymer; R'' and R' = sugar-polymer or Fuc.

FIG. 35T

119/498

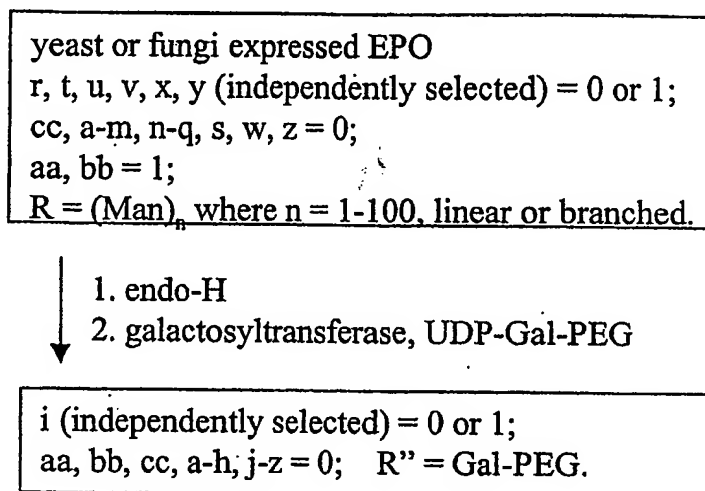


FIG. 35U

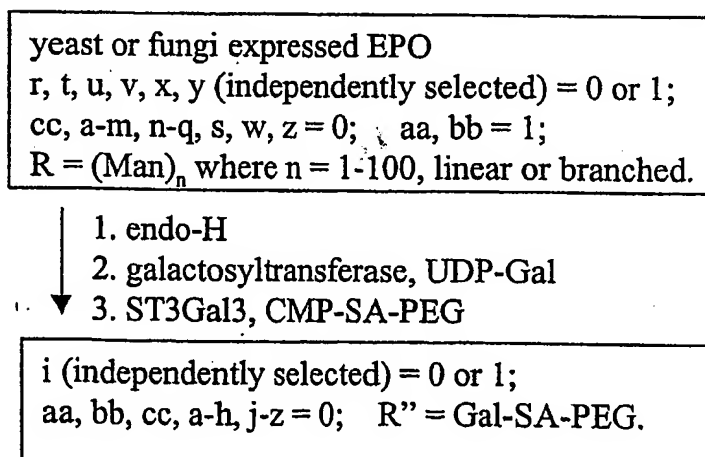


FIG. 35V

120/498

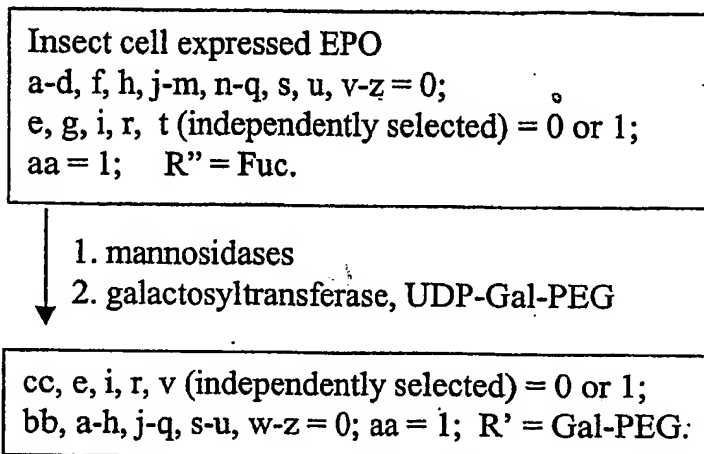
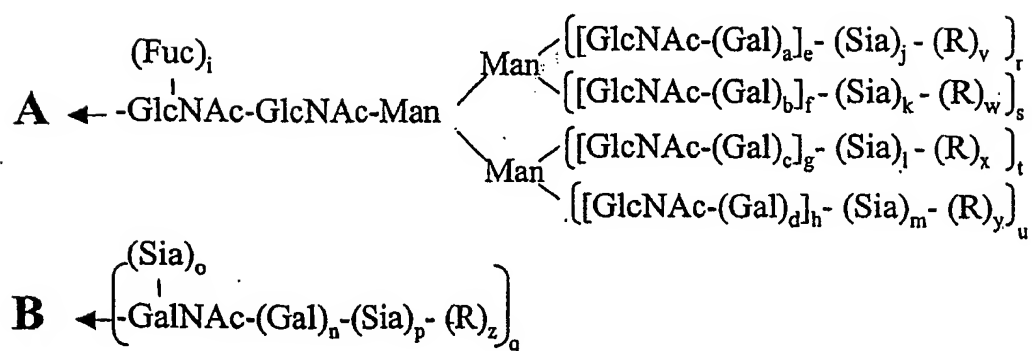
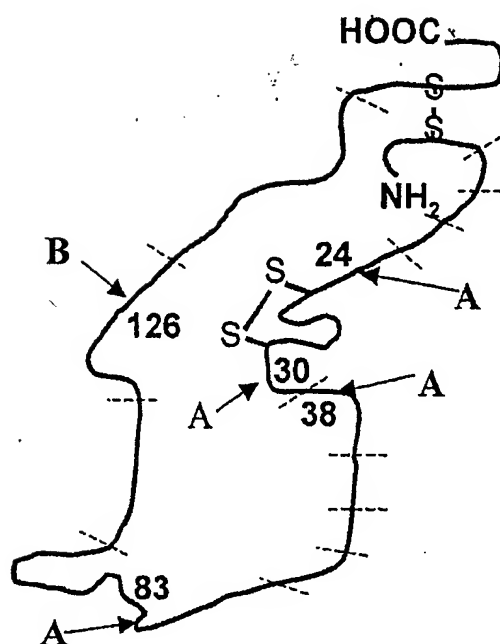


FIG. 35W

121/498



a-d, i, n-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 to 20.

v-z = 0;

R = polymer.

FIG. 35X



122/498

NSO expressed NESP  
 $q = 1$ ; a-i, n, r-u (independently selected) = 0  
 or 1; j-m, o, p, v-z = 0

- ↓
1. CMP-SA-levulinate, ST3Gal3,  
buffer, salt
  2.  $H_4N_2$ -PEG

$q = 1$ ; a-i, j-n, r-y (independently selected) =  
 0 or 1;  
 o, p, z = 0; R = PEG.

FIG. 35Y

CHO, BHK, 293 cells, Vero expressed NESP  
 a-g, n,  $q = 1$ ; h = 1 to 3;  
 j-m, i, o, p (independently selected) = 0 or 1;  
 r-u (independently selected) = 0 or 1;  
 v-z = 0

- ↓
1. CMP-SA-PEG,  $\alpha 2,8$ -ST

a-g, n,  $q = 1$ ; h = 1 to 3;  
 i, o, p (independently selected) = 0 or 1;  
 r-u (independently selected) = 0 to 1;  
 j-m (independently selected) = 0 to 2;  
 v-y (independently selected) = 1,  
 when j-m (independently selected) is 2;  
 R = PEG; z = 0.

FIG. 35Z

123/498

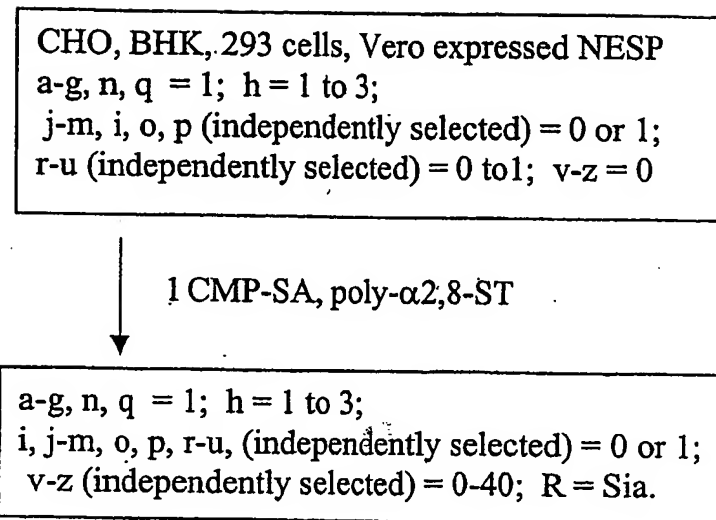
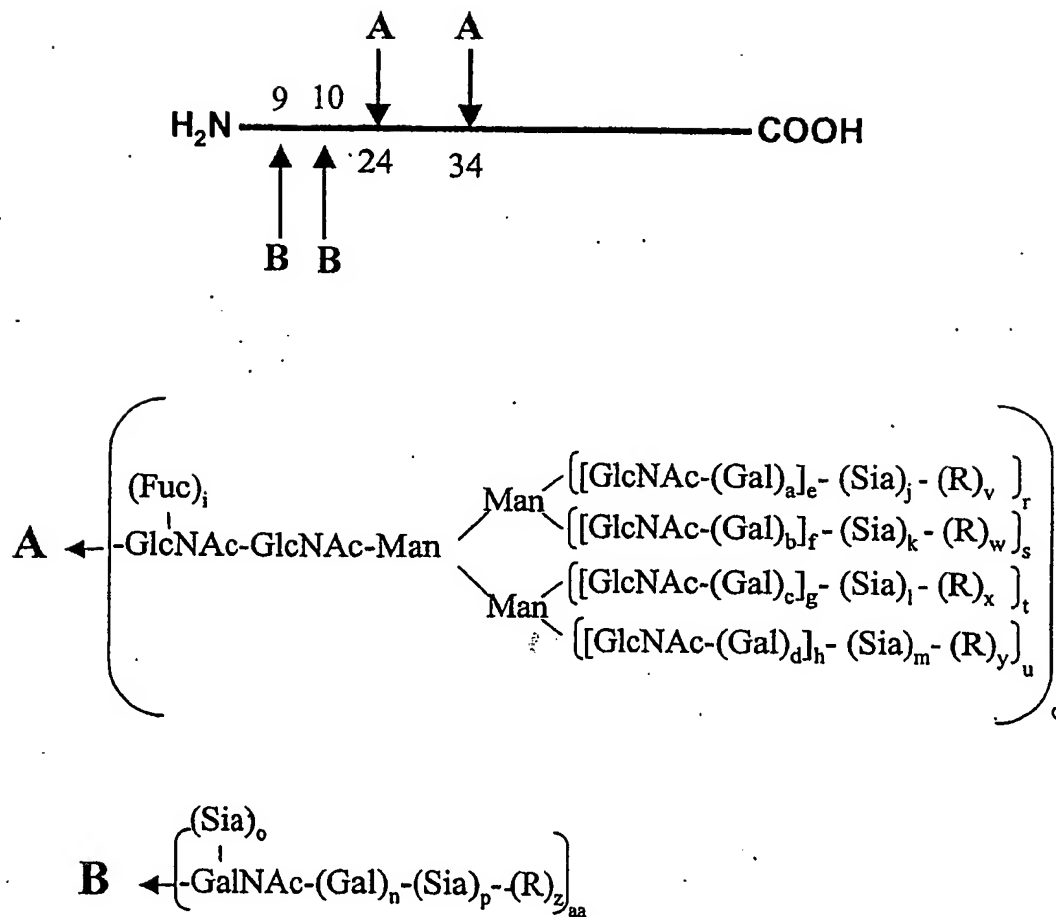


FIG. 35AA

124/498



a-d, i, n-u, aa (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = polymer, glycoconjugate.

FIG. 36A

125/498

CHO, BHK, 293 cells, Vero expressed GM-CSF.  
a-d, i-m, o-u, aa (independently selected) = 0 or 1;  
n, e-h = 1; v-z = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG (16 mol eq),  
ST3Gal3

a-d, i-m, q-u, aa (independently selected) = 0 or 1;  
o, p, z = 0; n, e-h = 1;  
v-y (independently selected) = 1,  
when j-m (independently selected) is 1;  
R = PEG.

FIG. 36B

CHO, BHK, 293 cells, Vero expressed GM-CSF.  
a-d, i-m, o-u, aa (independently selected) = 0 or 1;  
n, e-h = 1; v-z = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG (1.2 mol eq),  
ST3Gal3
  3. CMP-SA (16 mol eq), ST3Gal3 &  
ST3Gal1

a-d, i-m, p-u, aa (independently selected) = 0 or 1;  
o, z = 0; n, e-h = 1;  
v-y (independently selected) = 0 or 1; R = PEG.

FIG. 36C

126/498

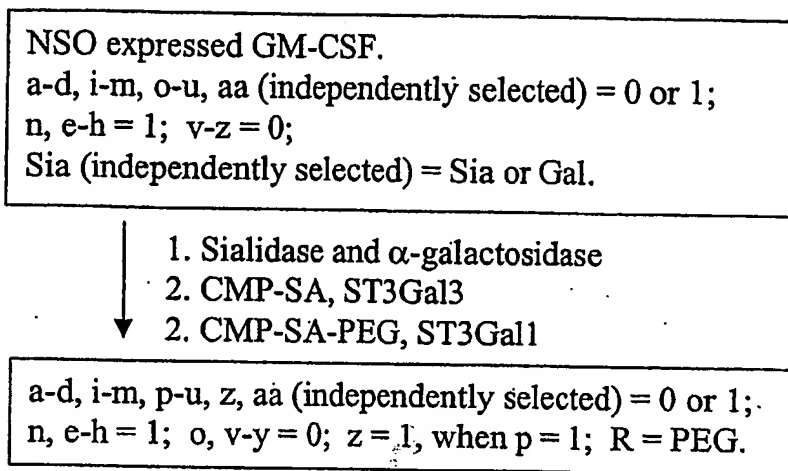


FIG. 36D

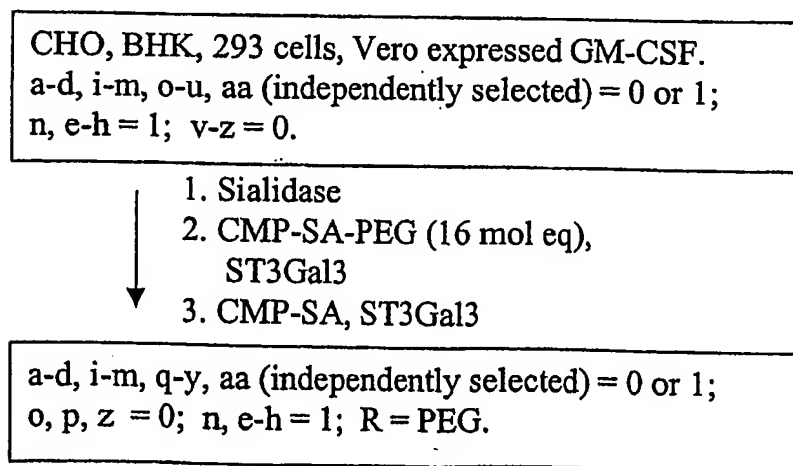


FIG. 36E

127/498

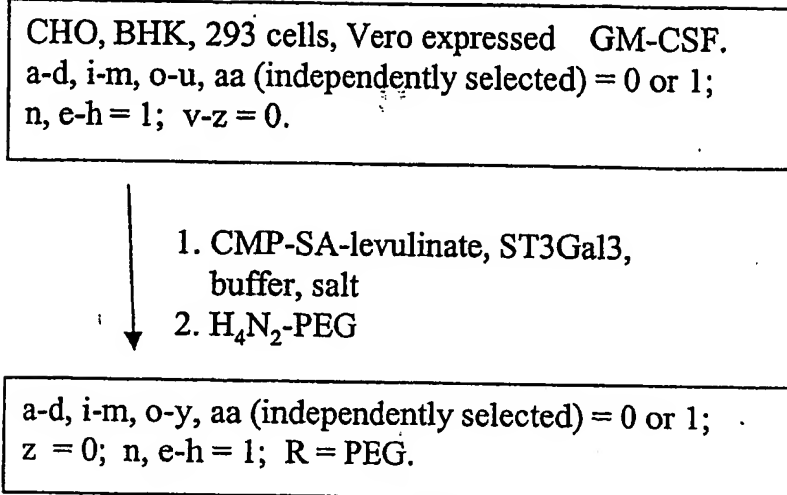


FIG. 36F

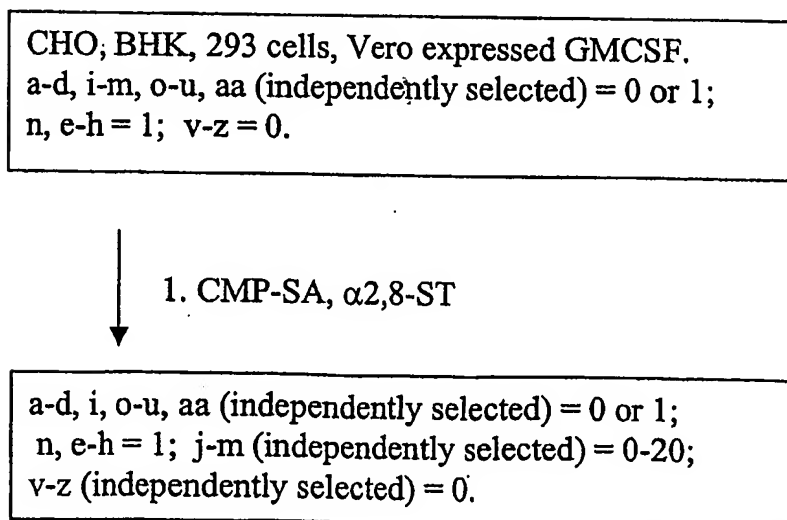
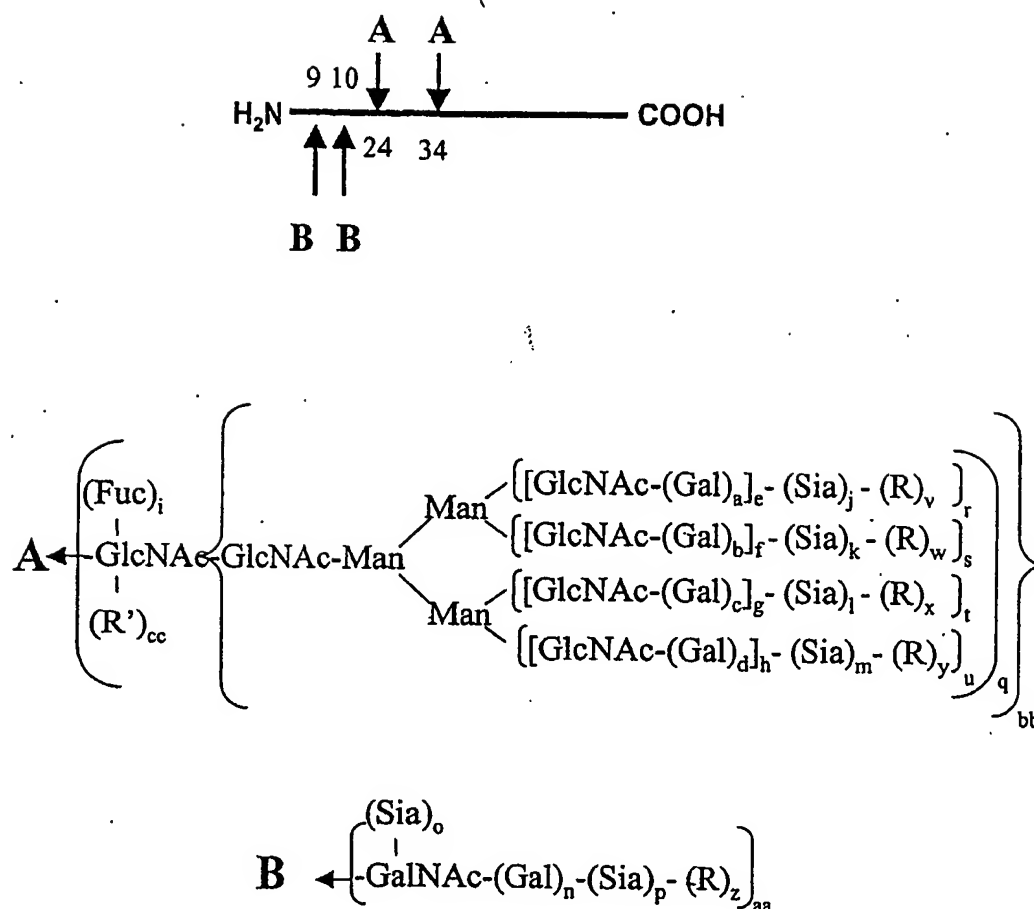


FIG. 36G

128/498



a-d, i, n-u, aa, bb, cc (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = modifying group, mannose, oligo-mannose.

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 36H

129/498

Insect cell expressed GM-CSF.

a-d, f, h, j-m, o, p, s, u, v-z = 0;

e, g, i, n, q, r, t, aa (independently selected) = 0 or 1.

- ↓
1. GNT's 1,2,4,5, UDP-GlcNAc
  2. Galactosyltransferase, UDP-Gal-PEG

a-i, n, q-u (independently selected) = 0 or 1;

j-m = 0; v-y (independently selected) = 1,

when e-h (independently selected) is 1;

R = PEG.

FIG. 36I

Yeast expressed GM-CSF.

a-p, z, cc = 0;

q-y, aa (independently selected) = 0 to 1;

bb = 1; R (branched or linear) = Man, oligomannose;

GalNAc = Man.

- ↓
1. Endoglycanase
  2. mannosidase (if aa = 1).
  3. Galactosyltransferase, UDP-Gal-PEG

a-p, r-z, aa, bb = 0;

q, cc (independently selected) = 0 or 1;

R' = -Gal-PEG.

FIG. 36J



130/498

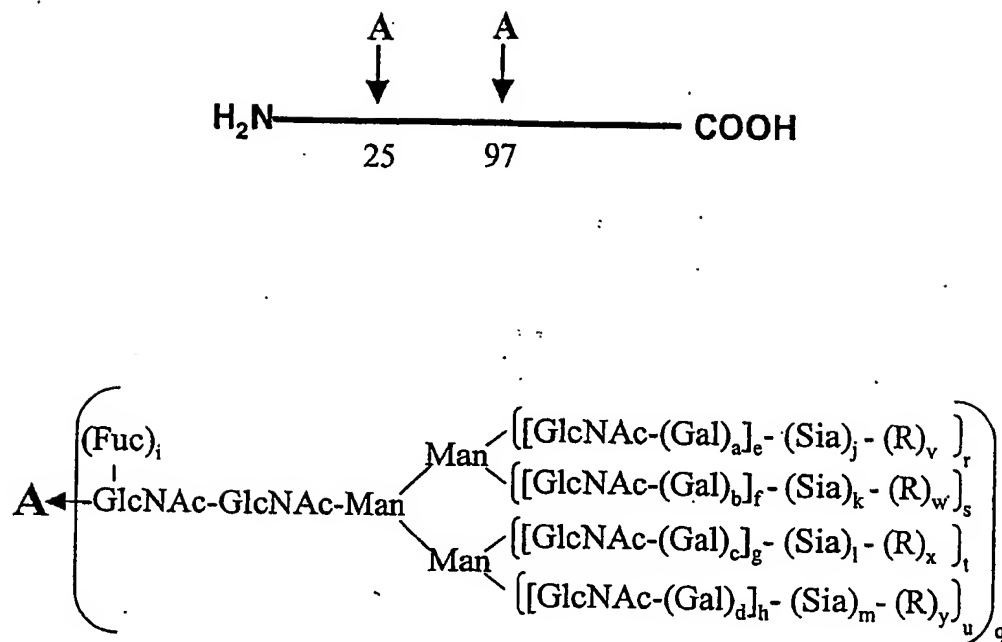
CHO, BHK, 293 cells, Vero expressed GM-CSF.  
a--m, o-u, aa, bb (independently selected) = 0 or 1;  
n, v-z, cc = 0.

- ↓
1. sialidase
  2. CMP-SA, ST3Gal3
  2. CMP-SA-linker-SA-CMP, ST3Gal1
  3. ST3Gal3, transferrin

a--m, p-u, z, aa (independently selected) = 0 or 1;  
o, v-y, cc = 0; bb, n = 1; R = transferrin.

FIG. 36K

131/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = polymer.

FIG. 37A

132/498

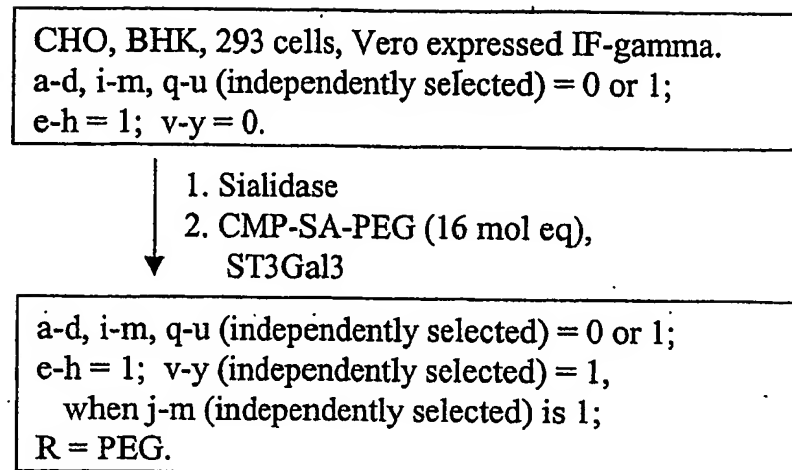


FIG. 37B

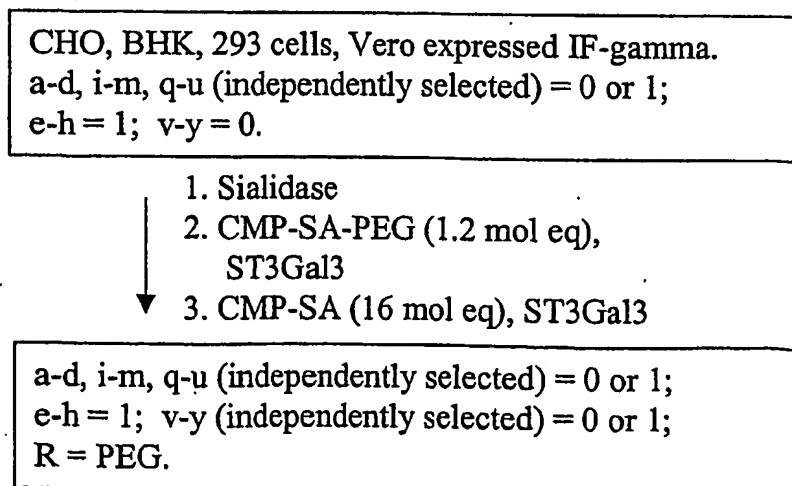


FIG. 37C

133/498

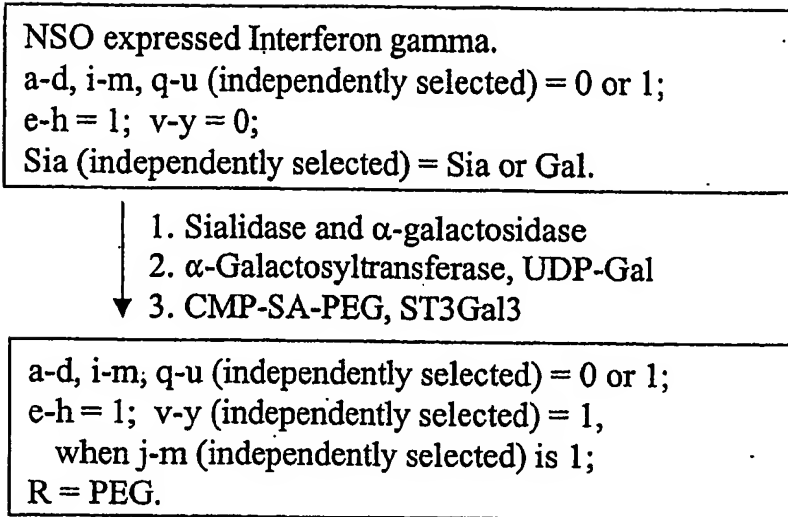


FIG. 37D

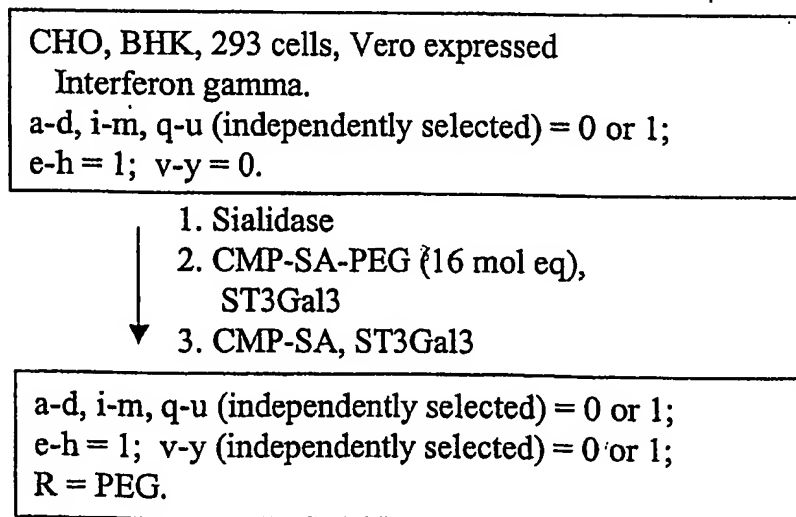


FIG. 37E

134/498

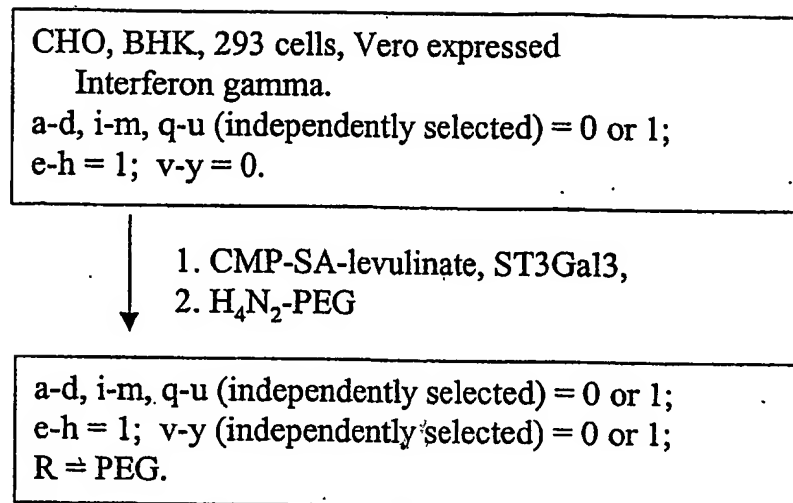


FIG. 37F

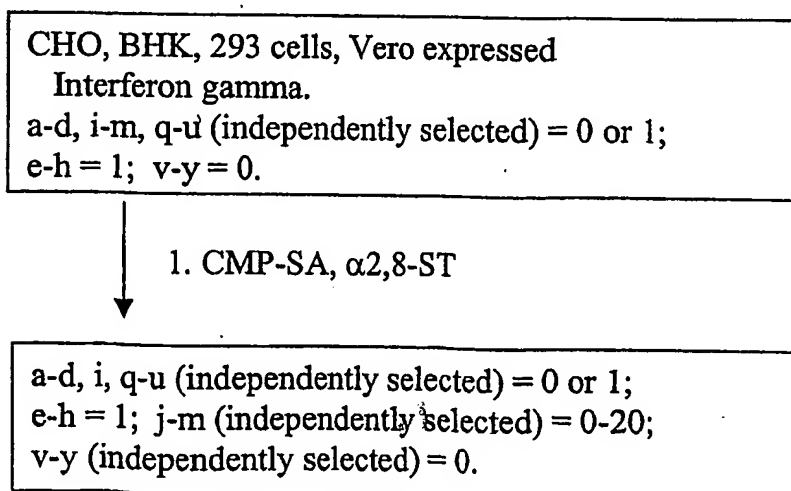
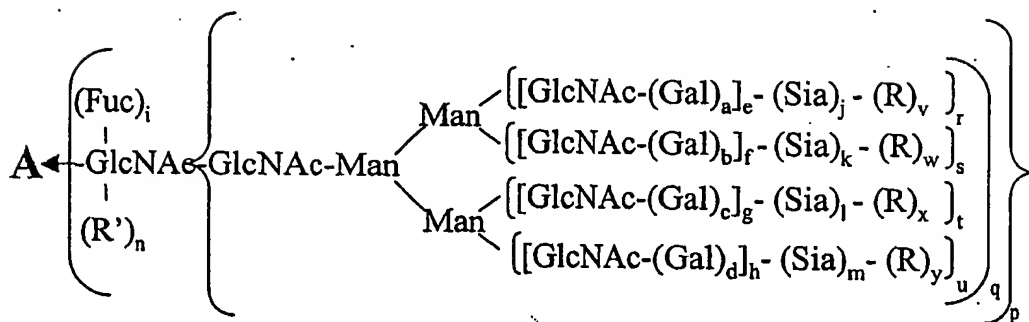
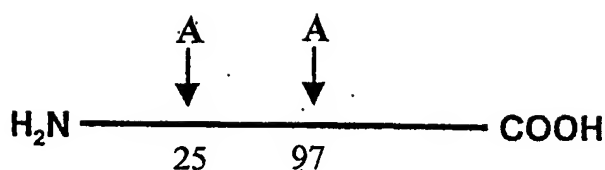


FIG. 37G

135/498



a-d, i, n, p-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0;

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group,  
glycoconjugate.

FIG. 37H

136/498

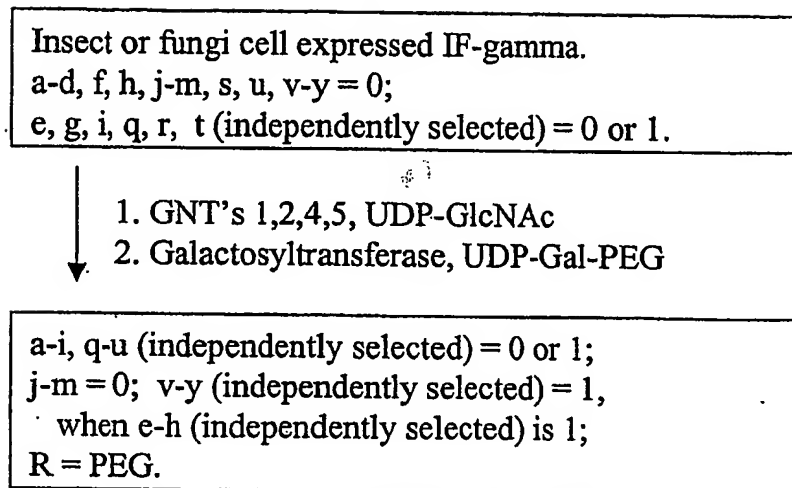


FIG. 37I

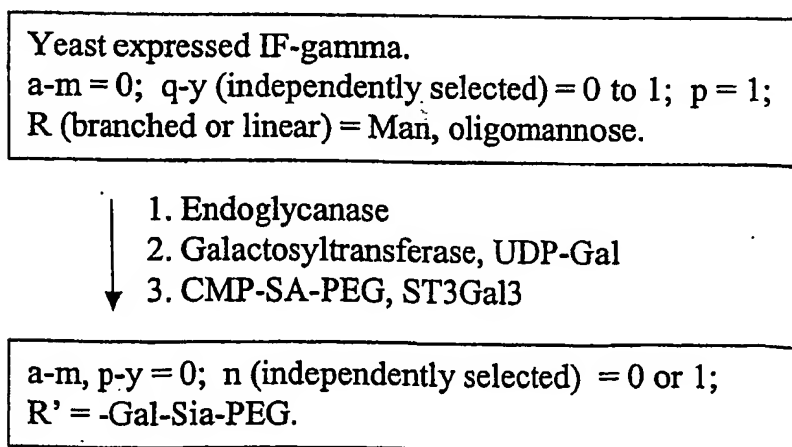


FIG. 37J

137/498

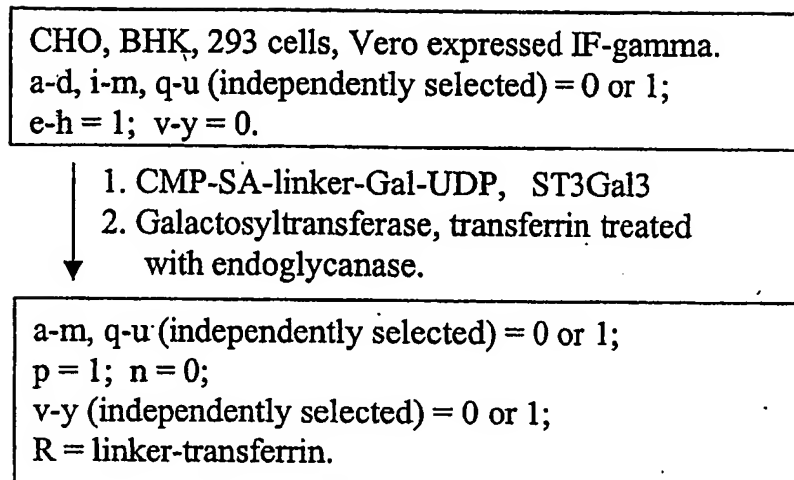


FIG. 37K

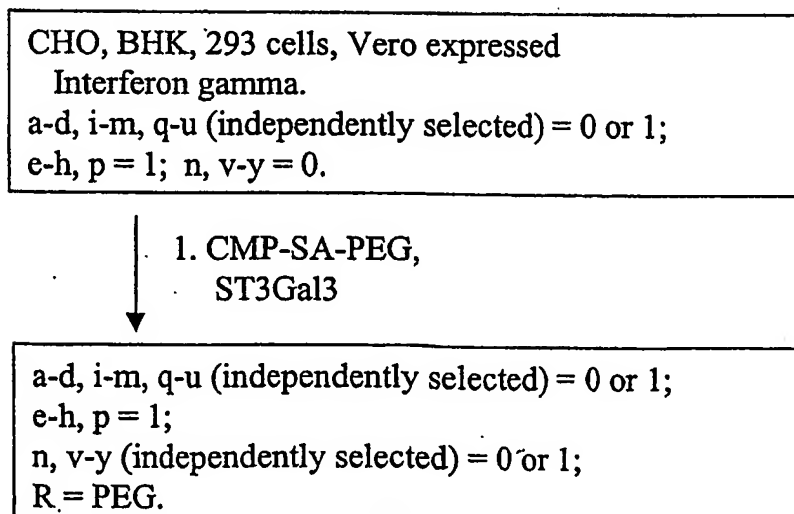


FIG. 37L



138/498

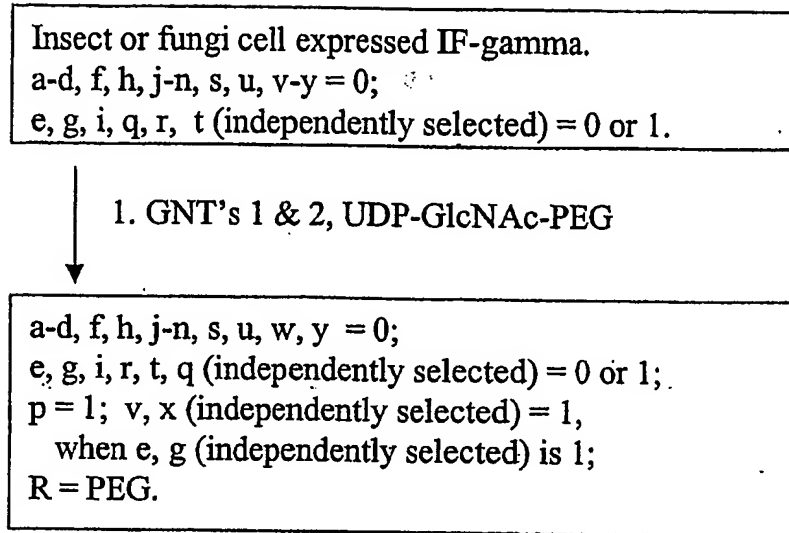


FIG. 37M

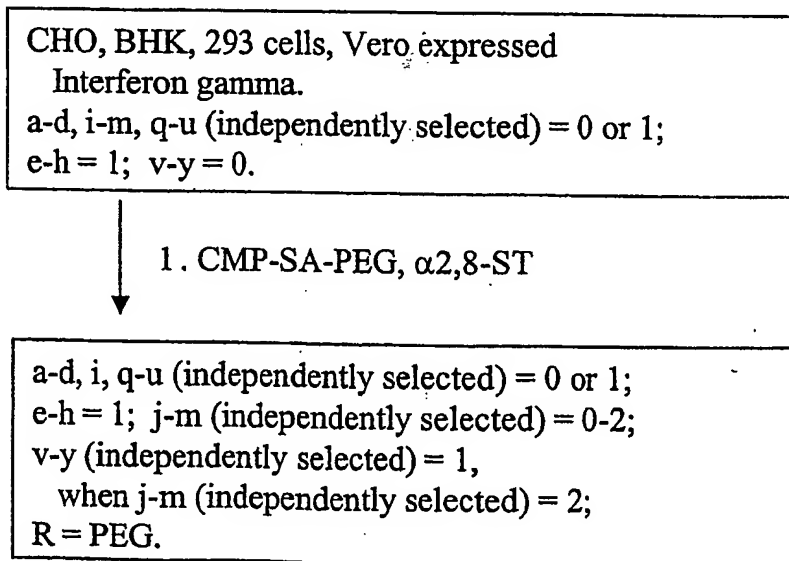


FIG. 37N



140/498

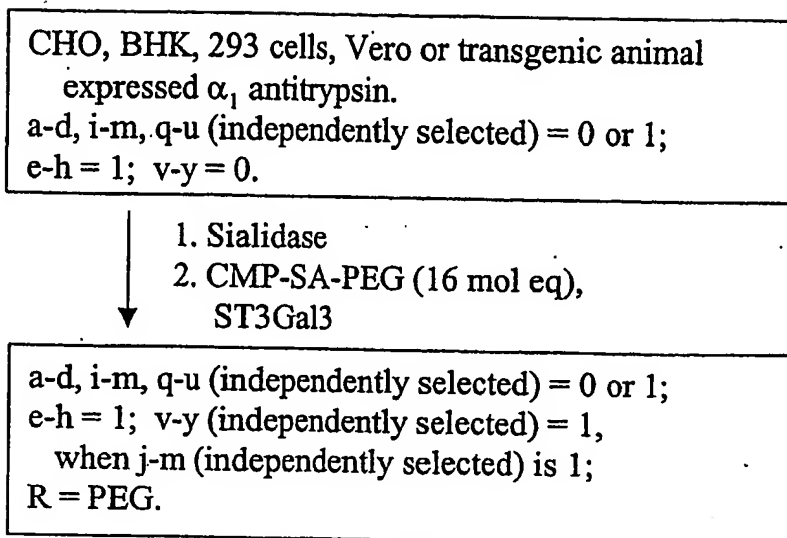


FIG. 38B

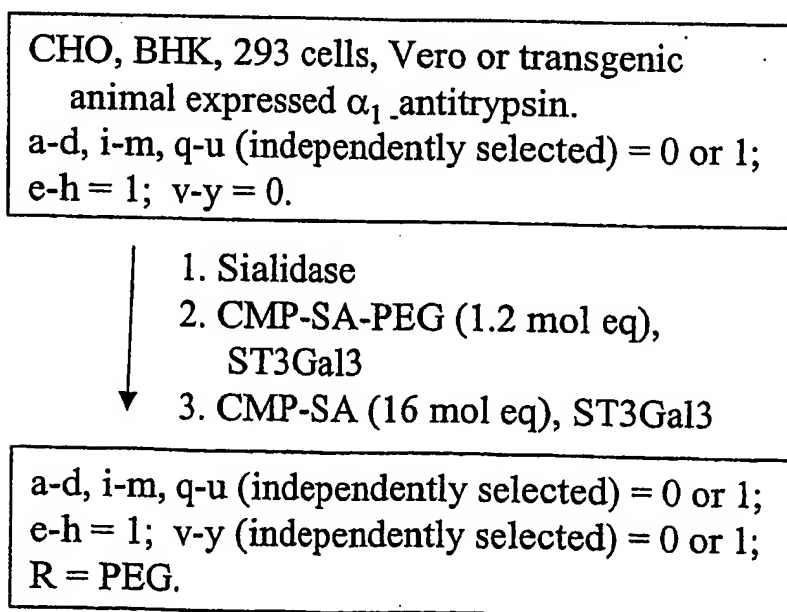


FIG. 38C

141/498

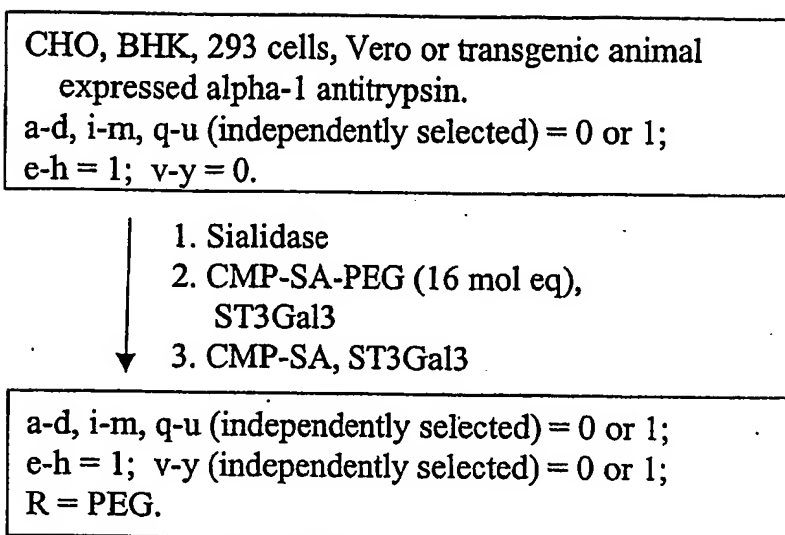


FIG. 38D

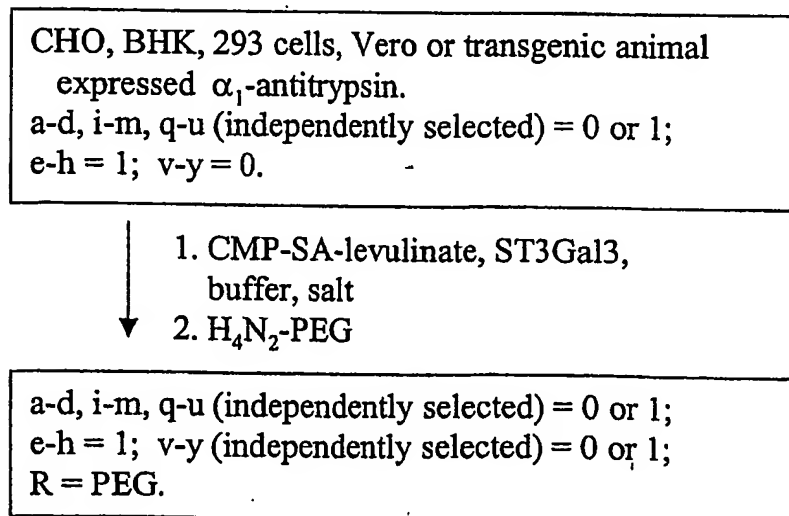


FIG. 38E

142/498

CHO, BHK, 293 cells, Vero expressed  $\alpha_1$ -antitrypsin.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

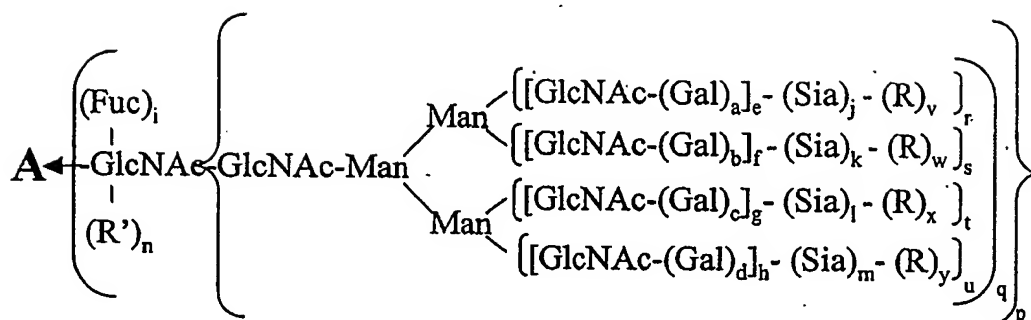
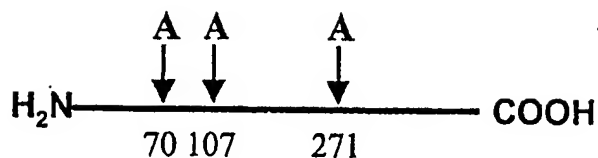


1. CMP-SA,  $\alpha$ 2,8-ST

a-d, i, q-u (independently selected) = 0 or 1; e-h = 1;  
j-m (independently selected) = 0-20;  
v-y (independently selected) = 0.

FIG. 38F

143/498



a-d, i, n, p-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0;

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 38G

144/498

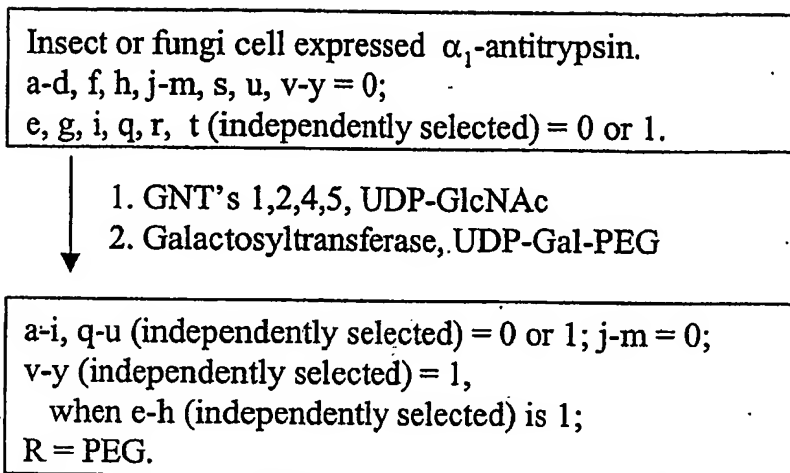


FIG. 38H

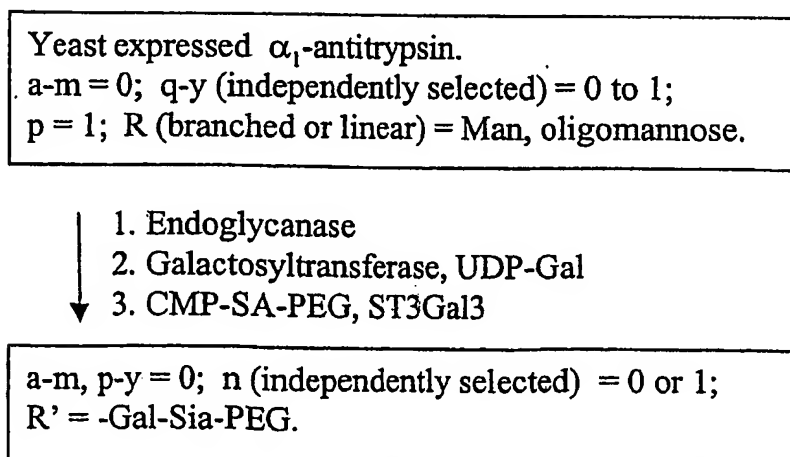


FIG. 38I

145/498

CHO, BHK, 293 cells, Vero expressed  $\alpha_1$ -antitrypsin.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

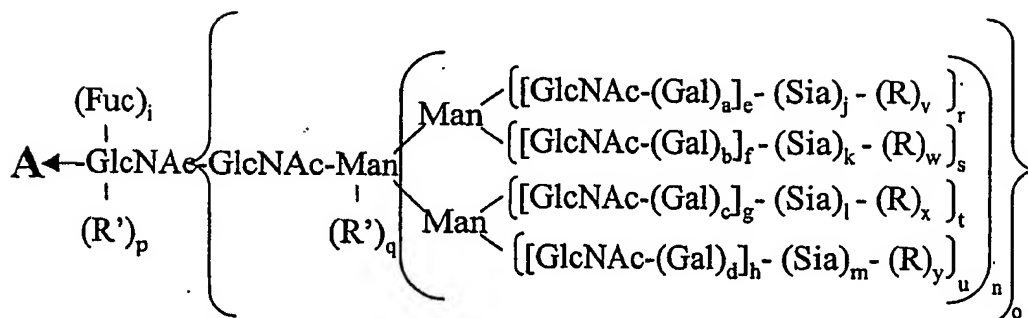
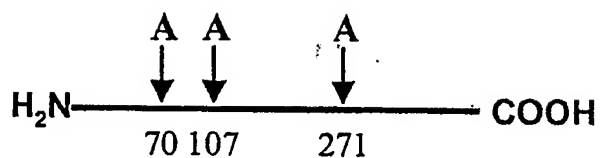
- ↓
1. CMP-SA-linker-Gal-UDP,  
ST3Gal3
  2. Galactosyltransferase, transferrin treated  
with endoglycanase

a-m, q-u (independently selected) = 0 or 1;  
p = 1; n = 0;  
v-y (independently selected) = 0 or 1;  
R = linker-transferrin.

FIG. 38J



146/498



a-d, i, n-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 to 20.

R = polymer;

R', R'' (independently selected) = sugar, glycoconjugate.

FIG. 38K

147/498

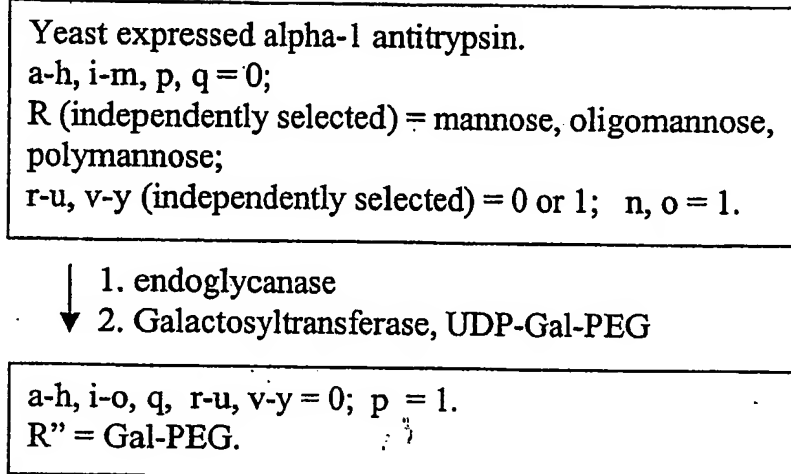


FIG. 38L

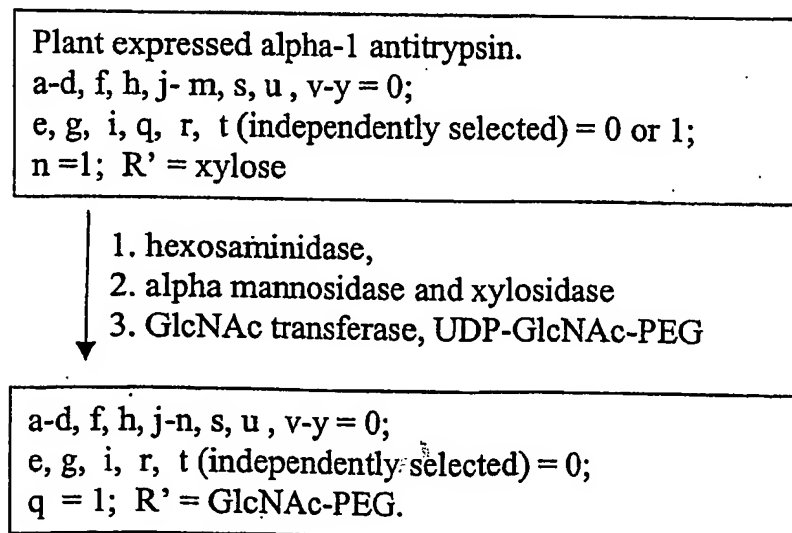


FIG. 38M

148/498

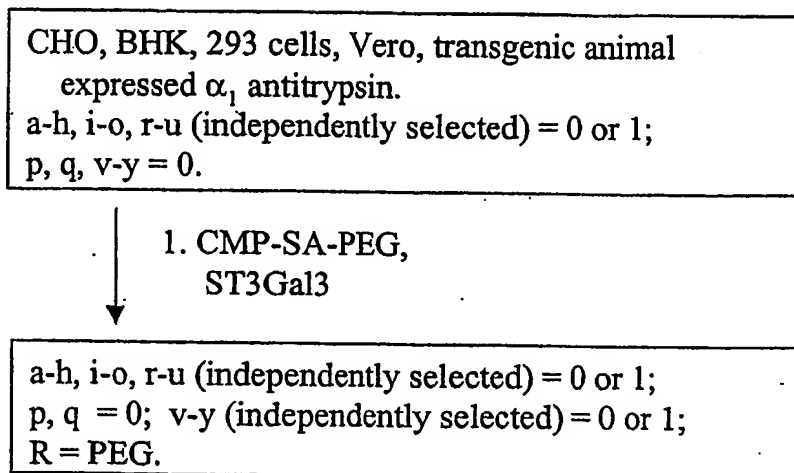
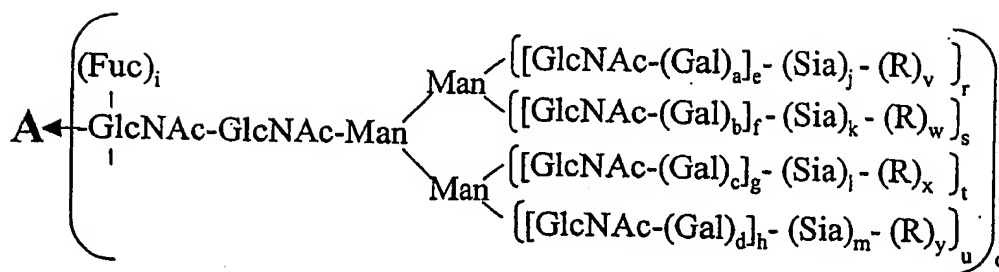
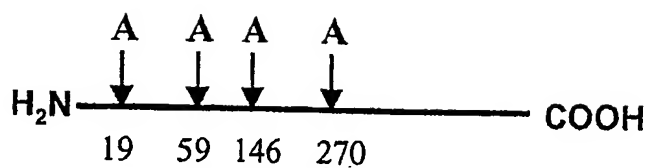


FIG. 38N

149/498



a-d, i, q-u (independently selected) = 0 or 1.  
 e-h (independently selected) = 0 to 6.  
 j-m (independently selected) = 0 to 100.  
 v-y = 0; R = polymer.

FIG. 39A

150/498

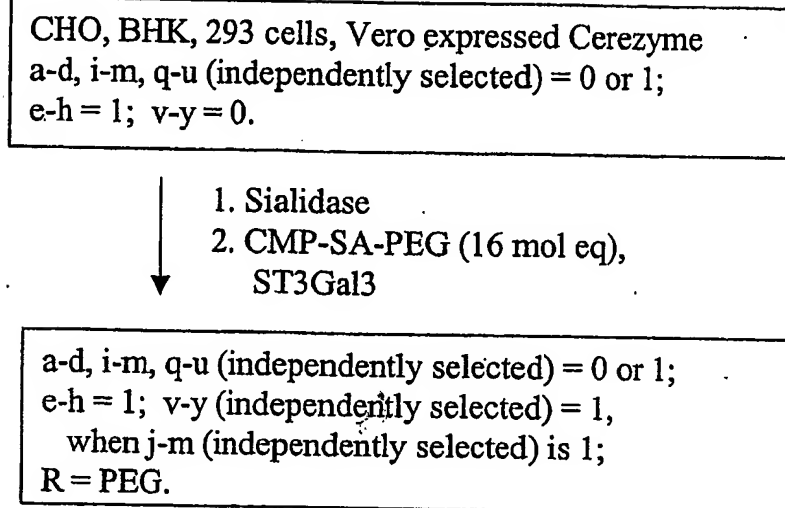


FIG. 39B

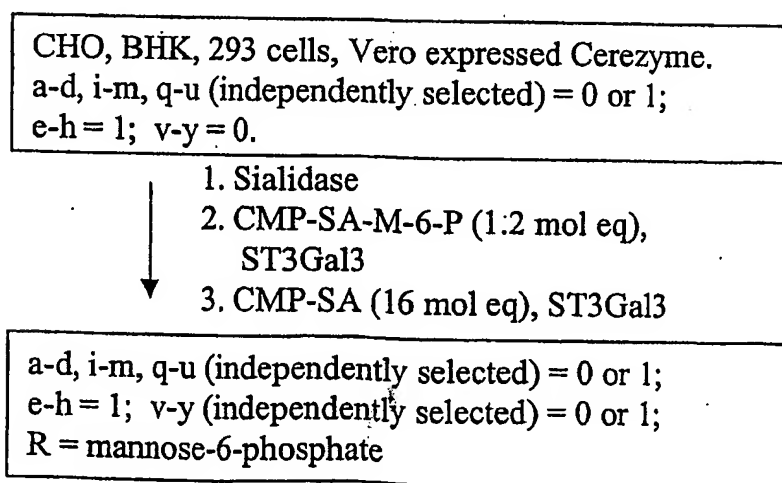


FIG. 39C

151/498

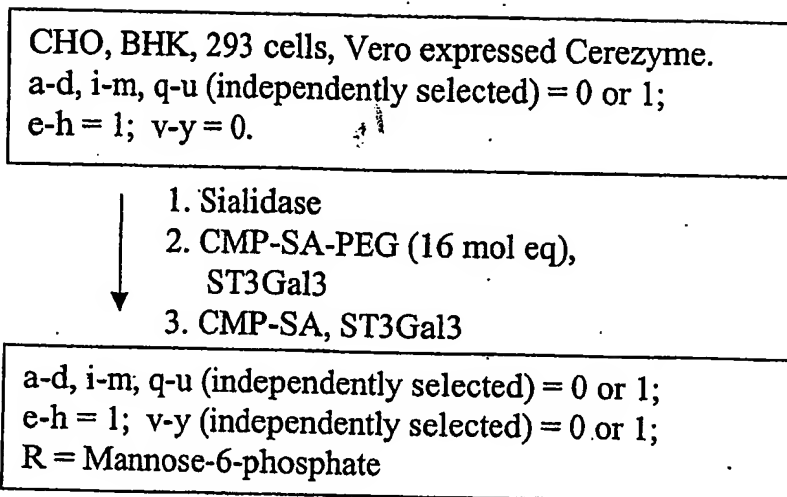


FIG. 39D

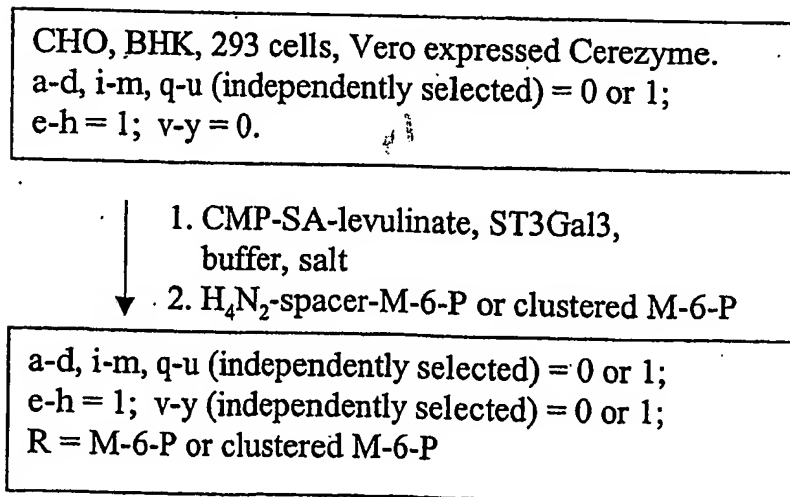


FIG. 39E

152/498

CHO, BHK, 293 cells, Vero expressed Cerezyme.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

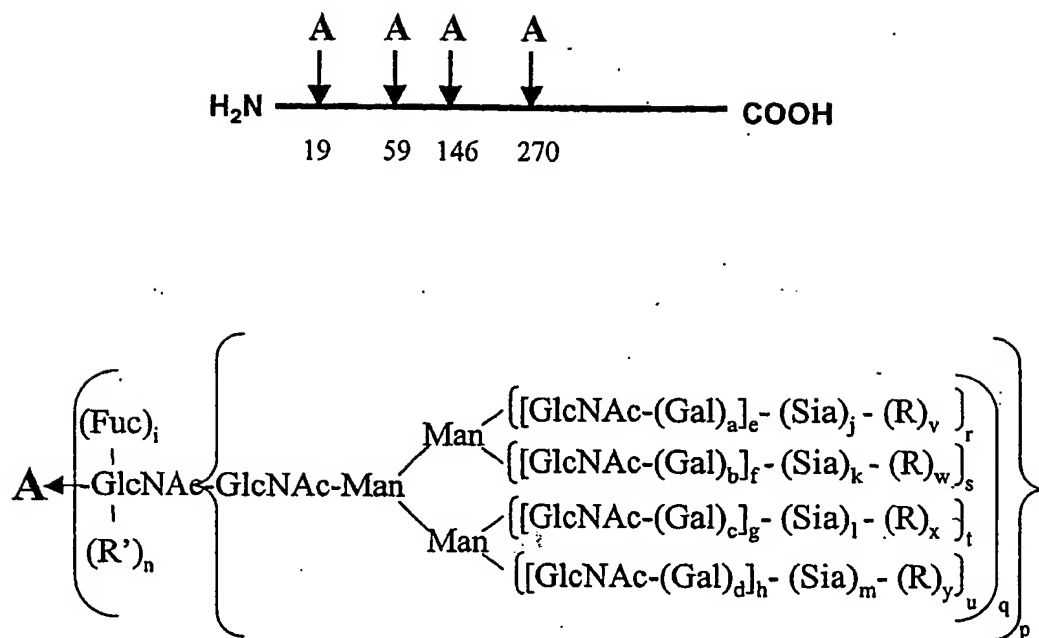


1. CMP-SA,  $\alpha$ 2,8-ST

a-d, i, q-u (independently selected) = 0 or 1;  
e-h = 1; j-m (independently selected) = 0-20;  
v-y (independently selected) = 0.

FIG. 39F

153/498



a-d, i, n, p-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0;

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group,  
glycoconjugate.

FIG. 39G



154/498

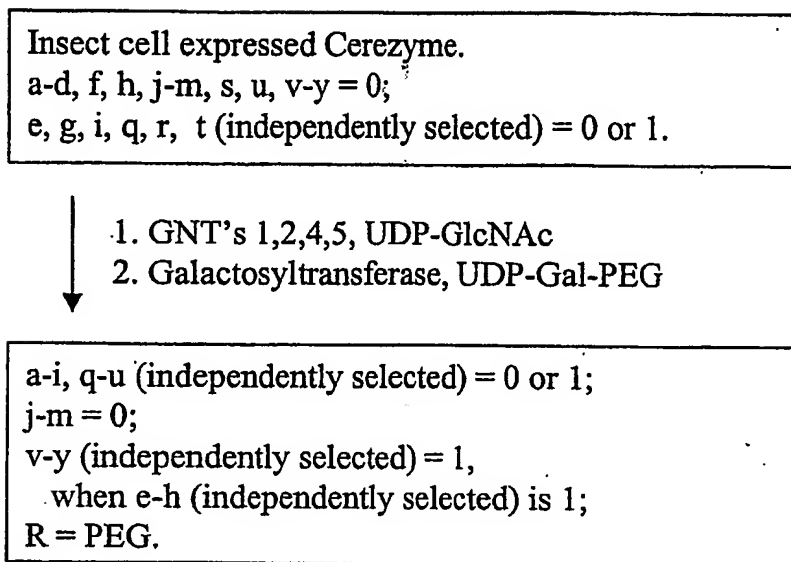


FIG. 39H

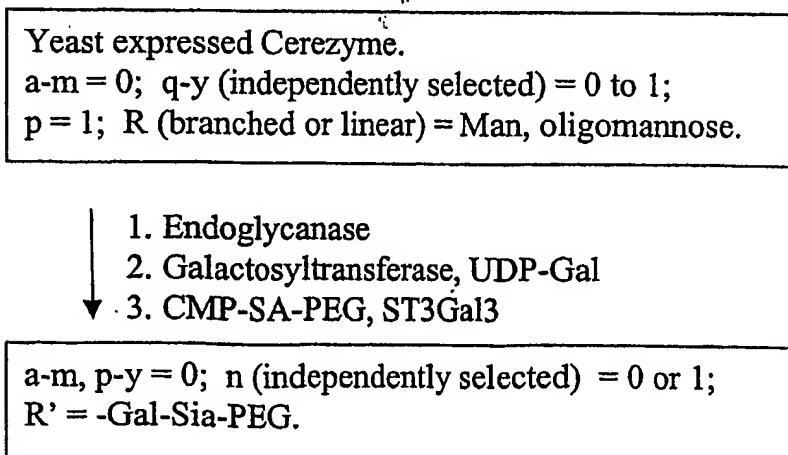



FIG. 39I

155/498

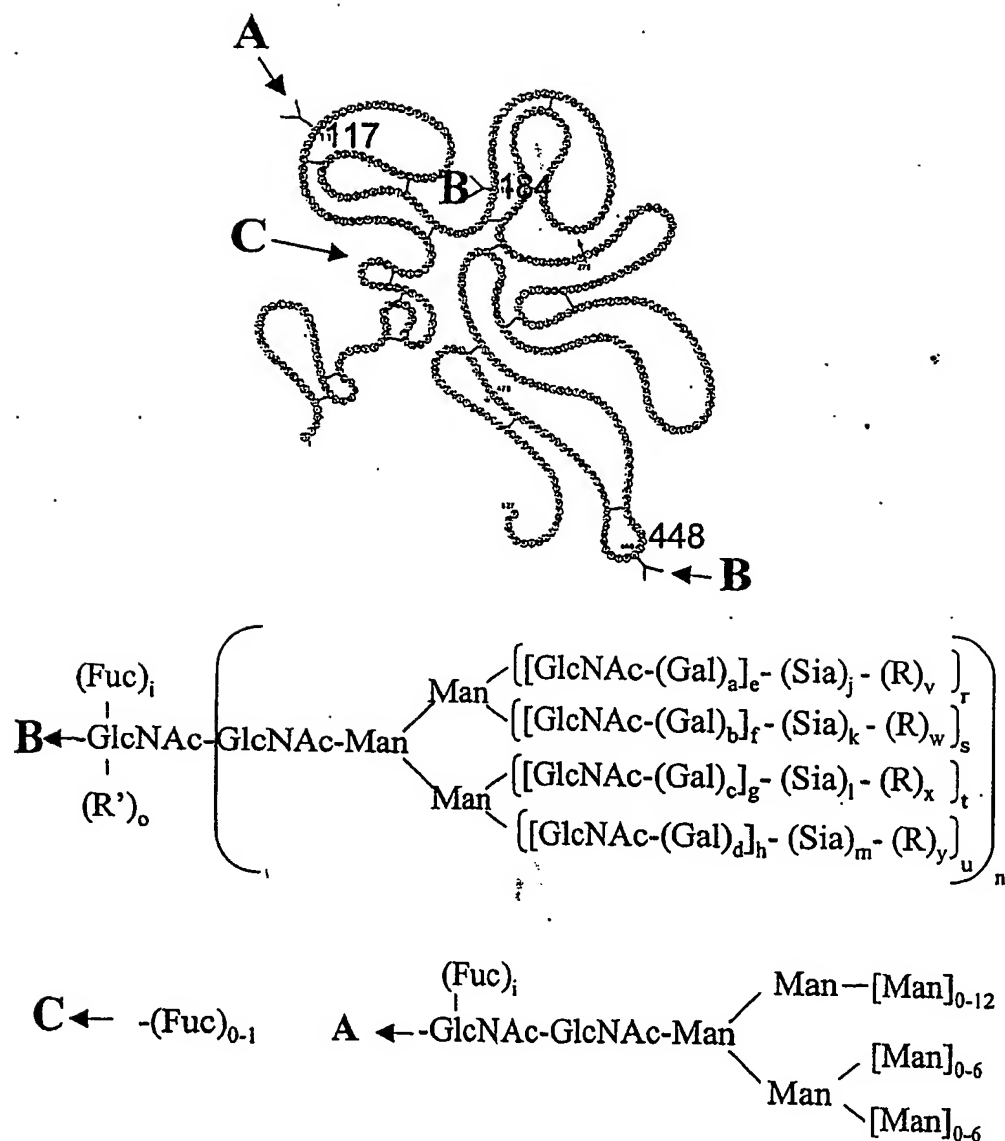
CHO, BHK, 293 cells, Vero expressed Cerezyme.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- 
1. CMP-SA-linker-SA-CMP,  
ST3Gal3
  2. ST3Gal3, desialylated transferrin.
  3. CMP-SA, ST3Gal3

a-m, q-u (independently selected) = 0 or 1;  
p = 1; n = 0; v-y (independently selected) = 0 or 1;  
R = linker-transferrin.

FIG. 39J

156/498



a-d, i, n-u (independently selected) = 0 or 1.  
 e-h (independently selected) = 0 to 4.  
 j-m (independently selected) = 0 to 20.  
 R = polymer; R' = sugar, glycoconjugate.

FIG. 40A

157/498

CHO, BHK, 293 cells, Vero expressed tPA  
 a-g, n = 1; h = 1 to 3;  
 j-m, i, (independently selected) = 0 or 1;  
 r-u (independently selected) = 0 to 1; o, v-y = 0.

1. Mannosidase(s), sialidase
2. GNT1,2 (4 and/or 5) UDP-GlcNAc
3. Gal transferase, UDP-Gal
4. CMP-SA-PEG, ST3Gal3

A = B; a-g, n = 1; h = 1 to 3;  
 i, r-u (independently selected) = 0 or 1;  
 o = 0; j-m, v-y (independently selected) = 0 or 1;  
 R = PEG

FIG. 40B

Insect or fungi cell expressed tPA  
 A = B; a-d, f, h, j-o, s, u, v-y = 0;  
 e, g, i, n, r, t (independently selected) = 0 or 1.

1. GNT's 1&2, UDP-GlcNAc
2. Galactosyltransferase, UDP-Gal
3. CMP-SA-PEG, ST3Gal3

A = B; b, d, f, h, k, m, o, s, u, w, y = 0;  
 a, c, e, g, i, r, t (independently selected) = 0 or 1;  
 n = 1; j, l, v, x (independently selected) = 0 or 1;  
 R = PEG.

FIG. 40C

158/498

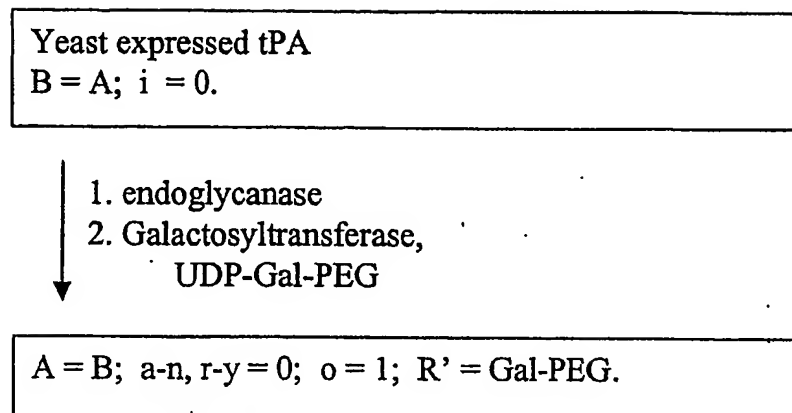


FIG. 40D

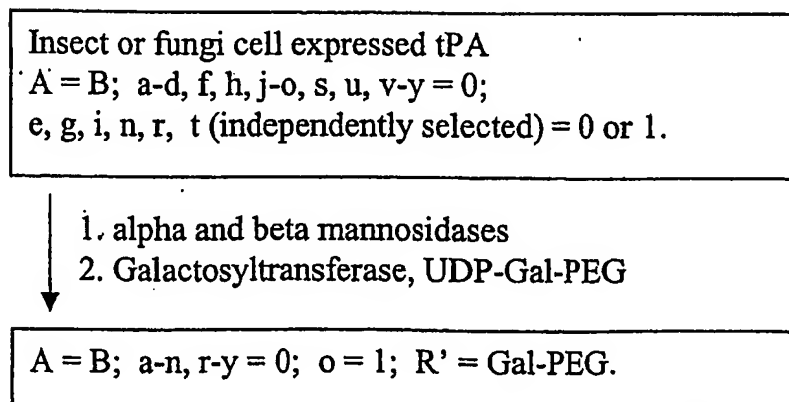


FIG. 40E

159/498

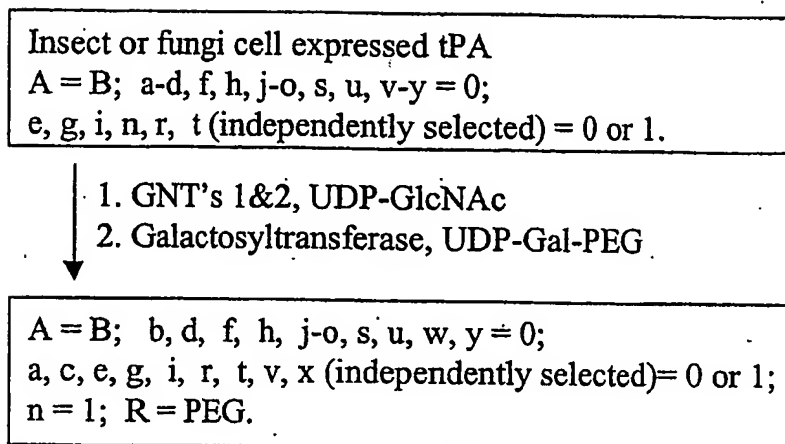


FIG. 40F

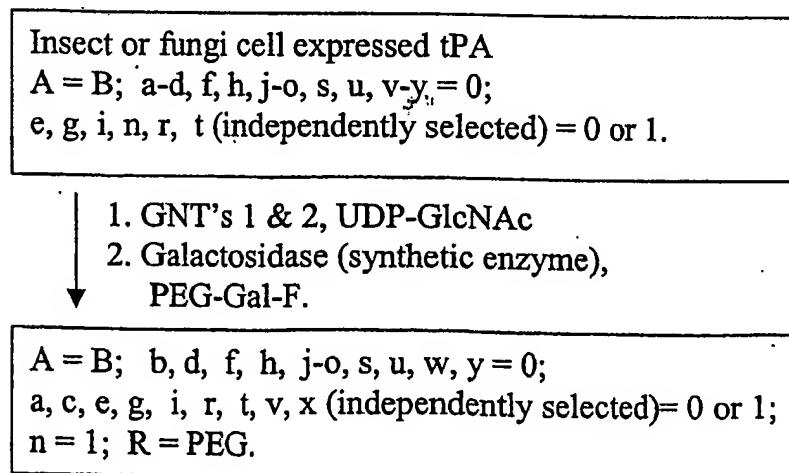
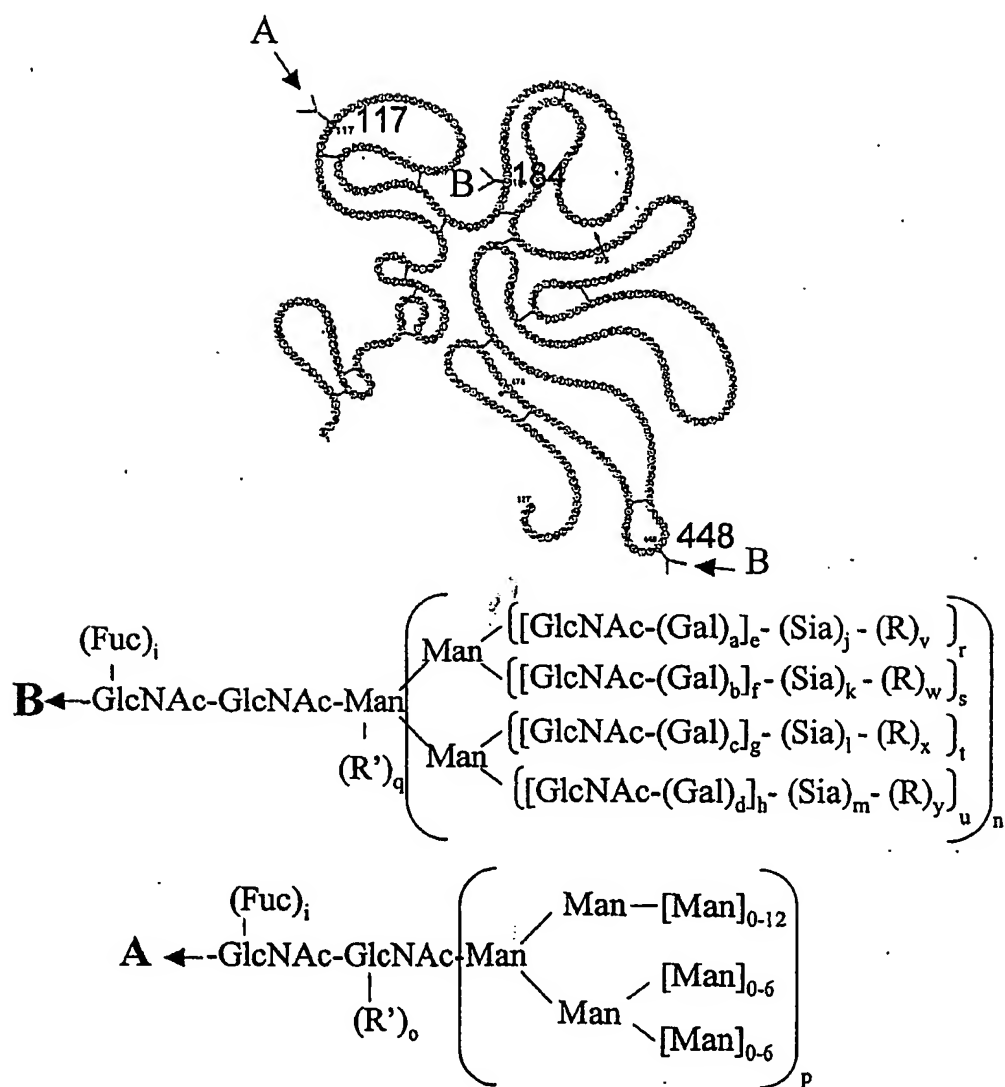


FIG. 40G

160/498



a-d, i, n-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 to 20.

R = polymer; R' = sugar, glycoconjugate.

FIG. 40H

161/498

NSO expressed tPA

A = B; a-m, r-u (independently selected) = 0 or 1;  
n = 1; o, p, q, v-y = 0

- ↓
1. sialidase, alpha-galactosidase
  2. CMP-SA-levulinate, ST3Gal3,
  3. H<sub>4</sub>N<sub>2</sub>-PEG

A = B; a-m, r-y (independently selected) = 0 or 1;  
n = 1; o, p, q = 0;  
v-y (independently selected) = 1,  
when j-m (independently selected) is 1;  
R = PEG.

FIG. 40I

CHO, BHK, 293 cells, Vero expressed tPA

a-g, n, p = 1; h = 1 to 3;  
j-m, i, (independently selected) = 0 or 1;  
r-u (independently selected) = 0 to 1; q, o, v-y = 0.

- ↓
1. alpha and beta Mannosidases
  2. CMP-SA, ST3Gal3
  3. Galactosyltransferase, UDP-Gal-PEG

a-g, n = 1; h = 1 to 3;  
i, r-u (independently selected) = 0 or 1; o = 1;  
q, p, v-y = 0; j-m (independently selected) = 0 or 1;  
R' = Gal-PEG

FIG. 40J



162/498

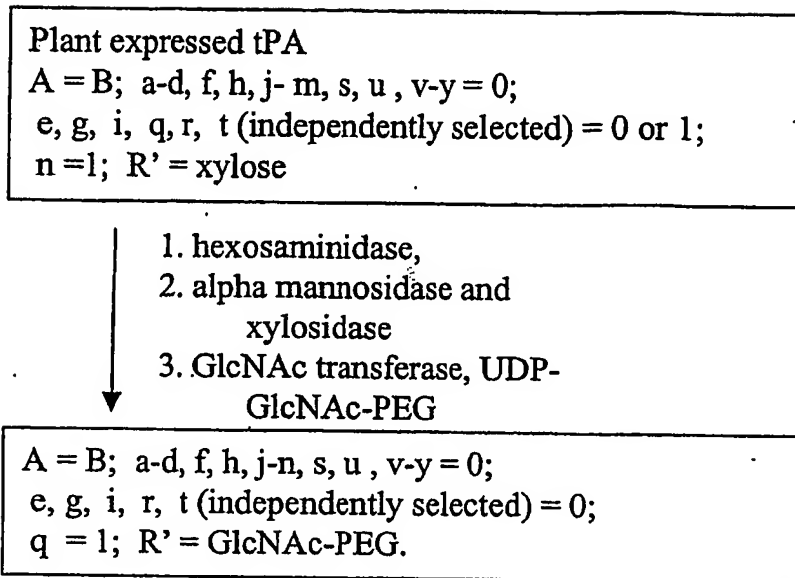
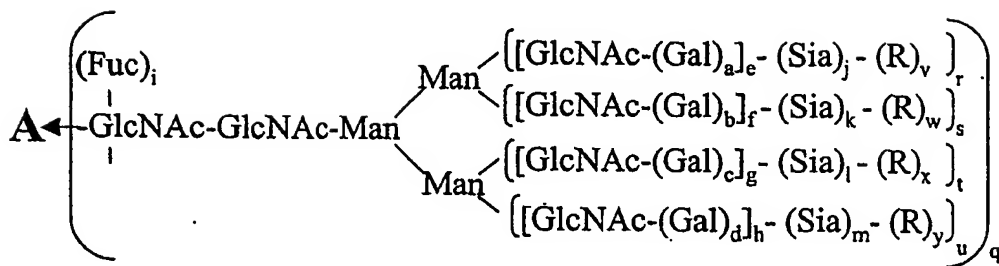
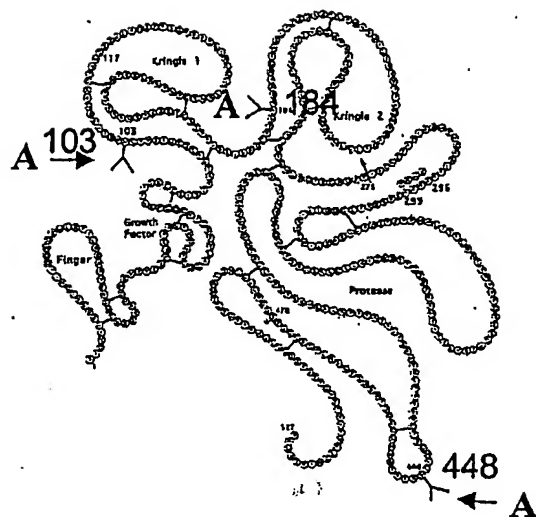


FIG. 40K

163/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = polymer.

FIG. 40L

164/498

CHO, BHK, 293 cells, Vero, expressed TNK tPA  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG (16 mol eq),  
ST3Gal3

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y (independently selected) = 1,  
when j-m (independently selected) is 1;  
R = PEG.

FIG. 40M

CHO, BHK, 293 cells, Vero expressed TNK tPA  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG (1.2 mol eq),  
ST3Gal3
  3. CMP-SA (16 mol eq), ST3Gal3

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y (independently selected) = 0 or 1;  
R = PEG.

FIG. 40N

165/498

NSO expressed TNK tPA

a-d, i-m, q-u (independently selected) = 0 or 1;

e-h = 1; v-y = 0;

Sia (independently selected) = Sia or Gal.

- ↓
1. Sialidase and  $\alpha$ -galactosidase
  2. Galactosyltransferase, UDP-Gal
  - ▼ 3. CMP-SA-PEG, ST3Gal3

a-d, i-m, q-u (independently selected) = 0 or 1;

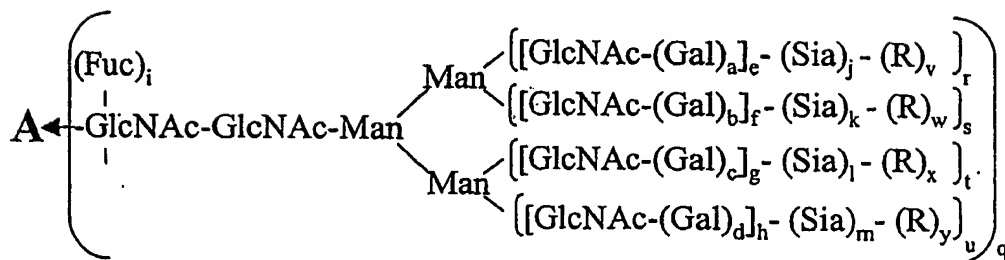
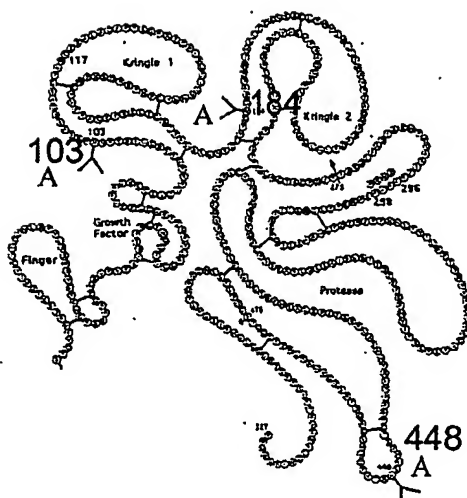
e-h = 1; v-y (independently selected) = 1,

when j-m (independently selected) is 1;

R = PEG.

FIG. 400

166/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

$$v-y=0; R = \text{polymer.}$$

FIG. 40P

167/498

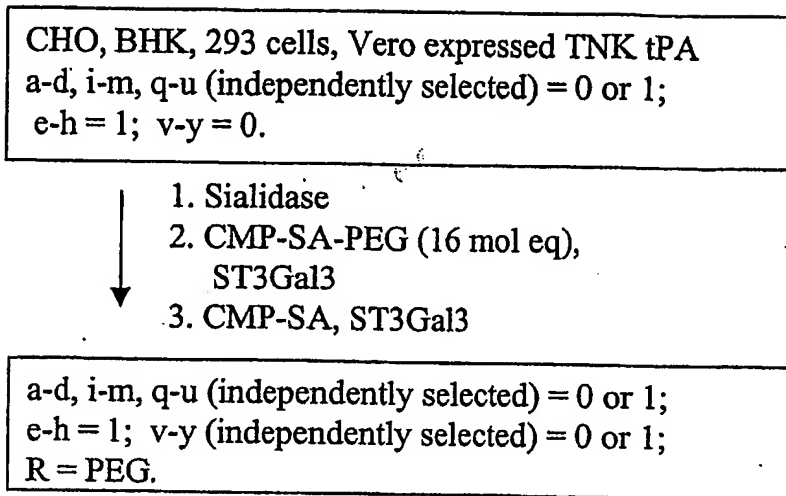


FIG. 40Q

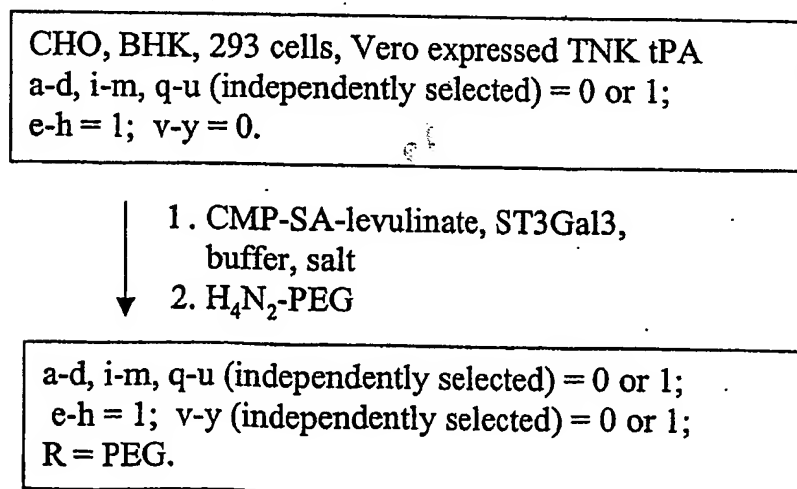


FIG. 40R

168/498

CHO, BHK, 293 cells, Vero expressed TNK tPA  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

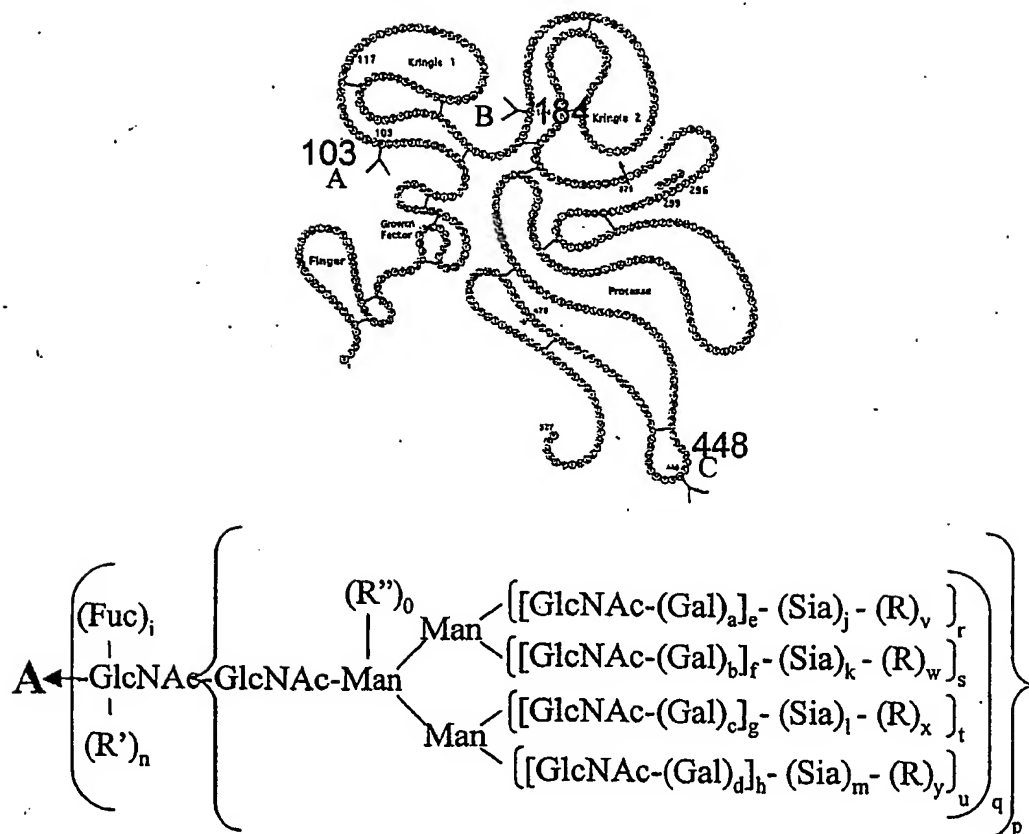


1. CMP-SA,  $\alpha$ 2,8-ST

a-d, i, q-u (independently selected) = 0 or 1;  
e-h = 1; j-m (independently selected) = 0-20;  
v-y (independently selected) = 0.

FIG. 40S

169/498



a-d, i, n-y (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group, glycoconjugate.

R'' = glycosyl residue.

FIG. 40T



170/498

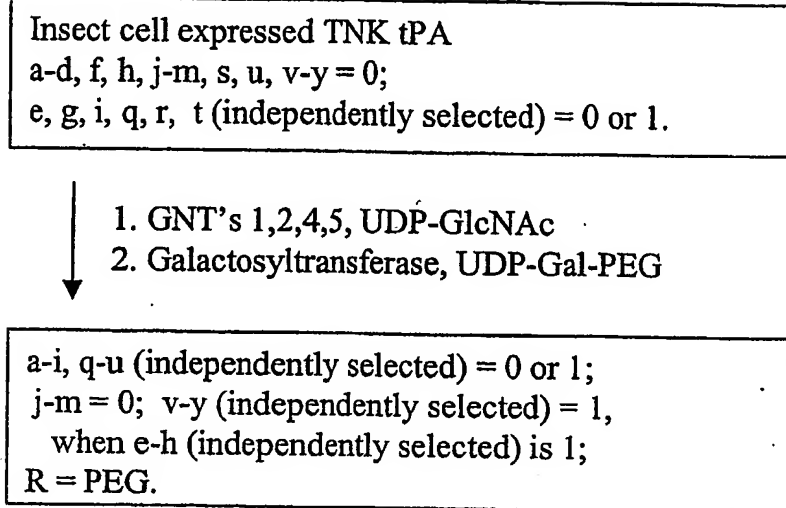


FIG. 40U

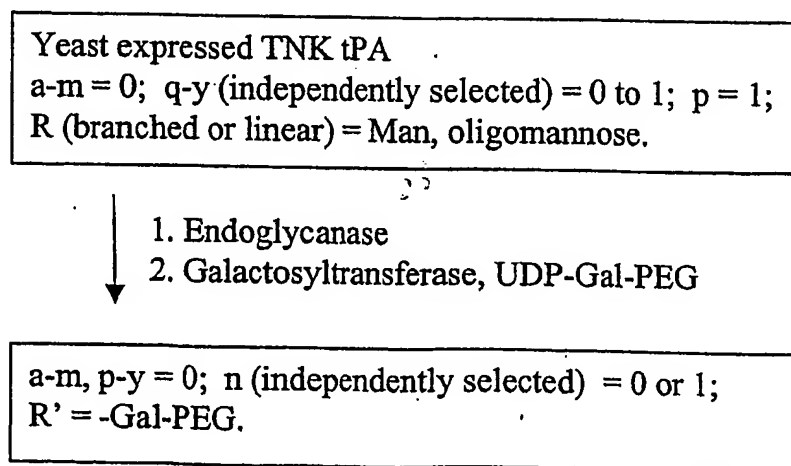


FIG. 40V

171/498

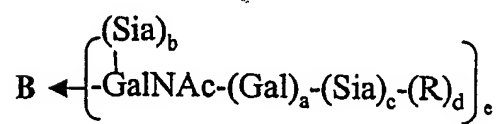
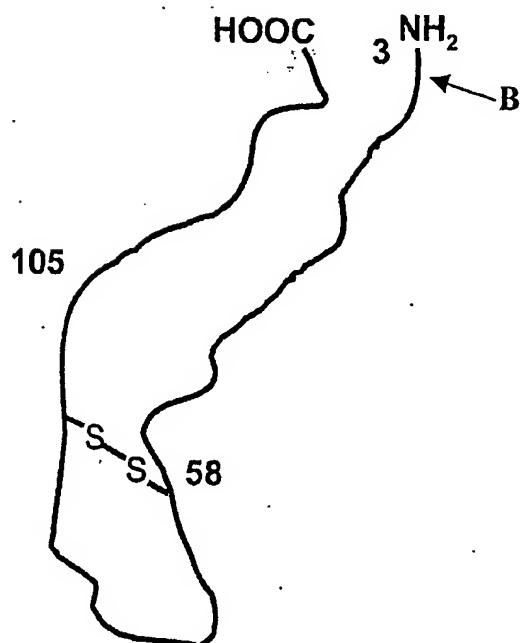
CHO, BHK, 293 cells, Vero expressed TNK tPA  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. CMP-SA-linker-Gal-UDP,  
ST3Gal3
  2. Galactosyltransferase, anti-TNF  
IG chimera produced in CHO.

a-m, r-u (independently selected) = 0 or 1; p, q = 1;  
n = 0; v-y (independently selected) = 0 or 1;  
R = linker-anti-TNF IG chimera protein.

FIG. 40W

172/498



a-c, e (independently selected) = 0 or 1;  
d = 0;

R = modifying group, mannose, oligo-mannose.

FIG. 41A

173/498

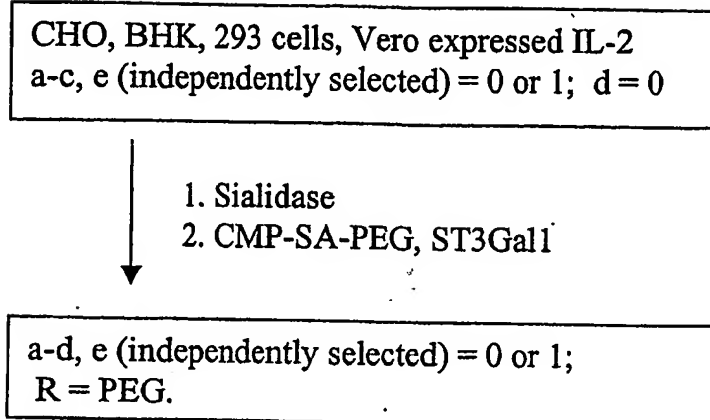


FIG. 41B

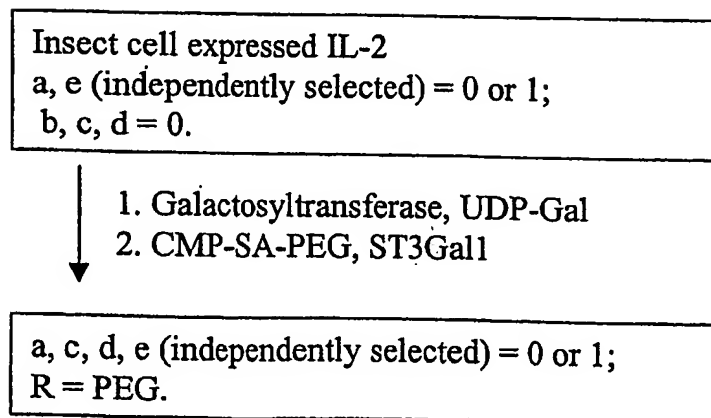


FIG. 41C

174/498

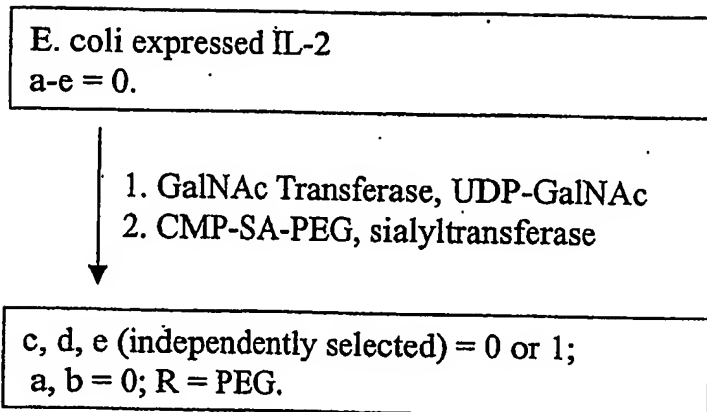


FIG. 41D

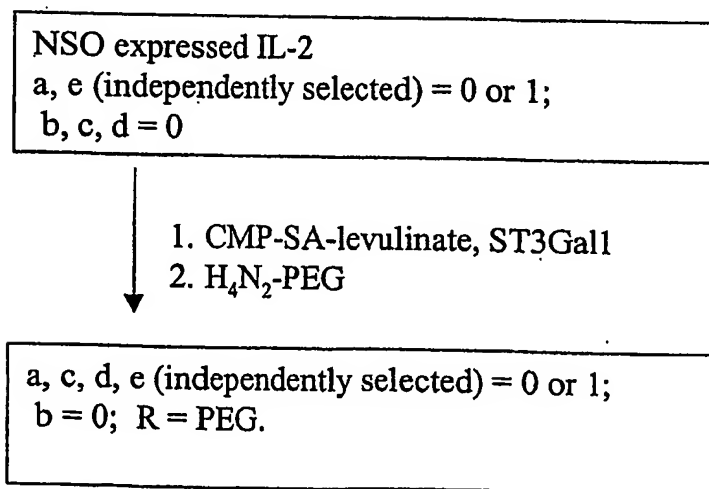


FIG. 41E

175/498

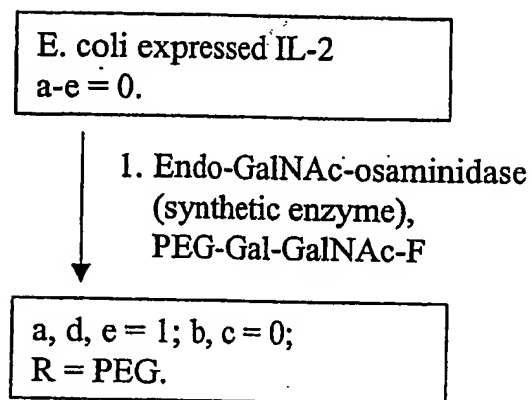


FIG. 41F

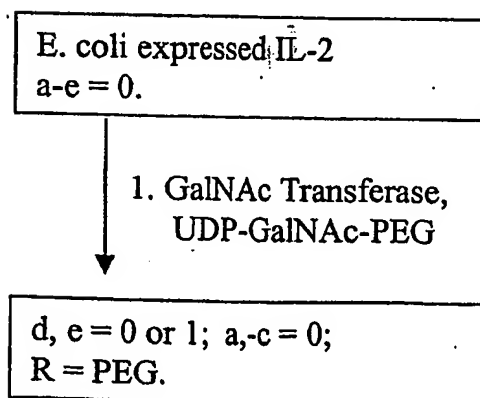
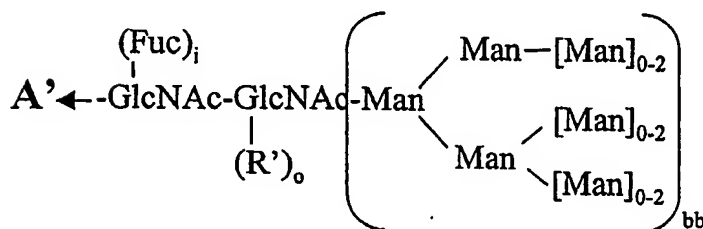
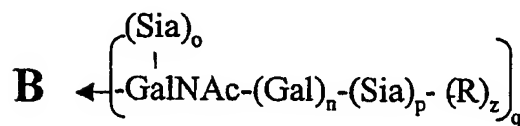
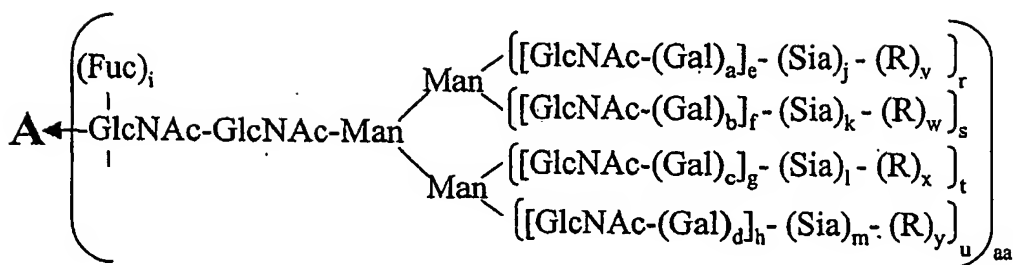


FIG. 41G

176/498

2 peptides  
 A and A' - N-linked sites  
 B - O-linked sites



Alternate structure  
 for some N-linked  
 structures of A.

a-d, i, n-u (independently selected) = 0 or 1.

aa, bb (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 20.

v-z = 0; R = polymer, glycoconjugate.

FIG. 42A

177/498

CHO, BHK, 293s cells, Vero, MDCK, HEKC expressed  
Factor VIII.

e-h = 1 to 4;

aa, bb, a-d, j-m, i, n-u (independently selected) = 0 or 1;

v-z = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG, ST3Gal3

e-h = 1 to 4;

aa, bb, a-d, i, n, q-u (independently selected) = 0 or 1;

o, p, z = 0; j-m, v-y (independently selected) = 0 or 1;

R = PEG.

FIG. 42B

CHO, BHK, 293S cells, Vero, MDCK, 293S, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, a-d, j-m, i, n-u (independently selected) = 0 or 1;

v-z = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG, ST3Gal3
  3. ST3Gal1, CMP-SA

e-h = 1 to 4;

aa, bb, a-d, i, n, p-u (independently selected) = 0 or 1;

o, z = 0; j-m, v-y (independently selected) = 0 or 1;

R = PEG.

FIG. 42C



178/498

CHO, BHK, 293s cells, Vero, MDCK, HEKC  
expressed Factor VIII.  
e-h = 1 to 4;  
aa, bb, a-d, j-m, i, n-u (independently selected)=0 or 1;  
v-z = 0.



1. CMP-SA-PEG, ST3Gal3

e-h = 1 to 4;  
aa, bb, a-d, i, n-u (independently selected) = 0 or 1;  
z = 0; j-m, v-y (independently selected) = 0 or 1;  
R = PEG.

FIG. 42D

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.  
e-h = 1 to 4;  
aa, bb, a-d, j-m, i, n-u (independently selected) 0 or 1;  
v-z = 0.



1. CMP-SA-PEG, ST3Gal1

e-h = 1 to 4;  
aa, bb, a-d, i, n-u (independently selected) = 0 or 1;  
z = 0; j-m, v-y (independently selected) = 0 or 1;  
R = PEG.

FIG. 42E

179/498

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, a-d, j-m, i, n-u (independently selected)=0 or 1;

v-z = 0.



1. CMP-SA-PEG,  $\alpha$ 2,8-ST

e-h = 1 to 4;

aa, bb, a-d, i, n-y (independently selected) = 0 or 1;

z = 0; j-m (independently selected) = 0 to 2;

v-y (independently selected) = 1,

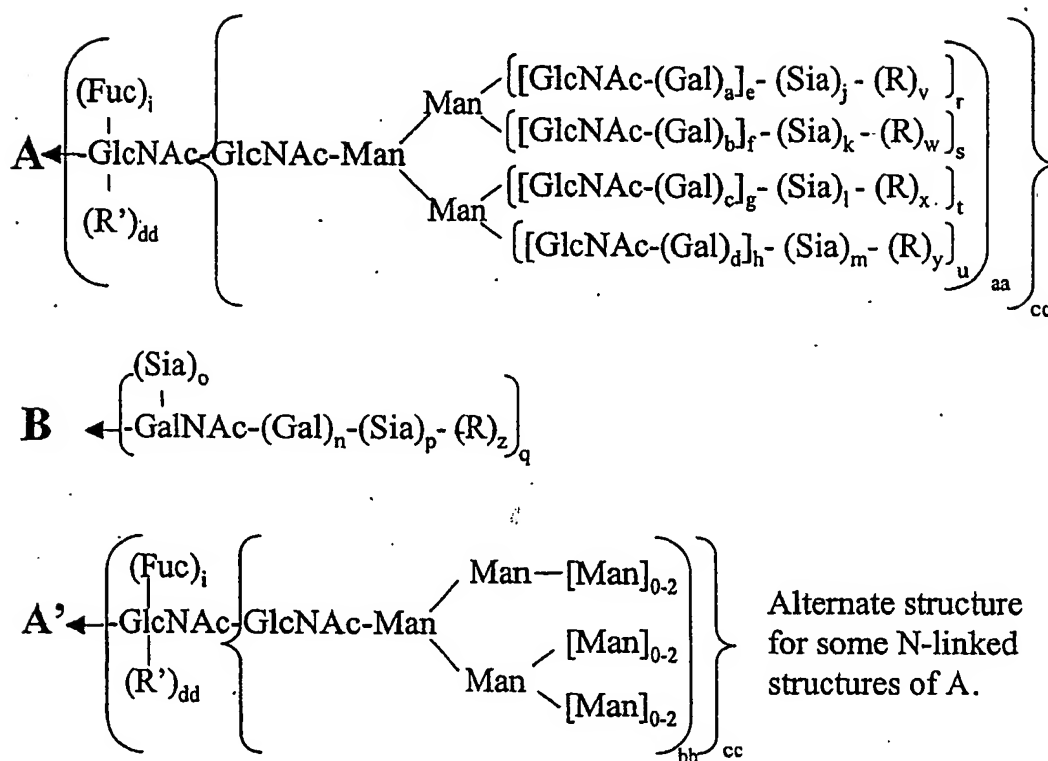
when j-m (independently selected) is 2;

R = PEG.

FIG. 42F

180/498

2 peptides  
**A or A'** - N-linked sites  
**B** - O-linked sites



a-d, i, n-u, (independently selected) = 0 or 1.

aa, bb, cc, dd (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 20.

v-z = 0;

R = modifying group, mannose, oligo-mannose.

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 42G

181/498

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, cc, a-d, j-m, i, n-u (independently selected) = 0 or 1;

dd, v-z = 0.

- ↓ 1. CMP-SA-levulinate, ST3Gal3,  
2. H<sub>4</sub>N<sub>2</sub>-PEG

e-h = 1 to 4;

aa, bb, cc, a-d, i, n-u (independently selected) = 0 or 1;

dd, z = 0; j-m, v-y (independently selected) = 0 or 1;

R = PEG.

FIG. 42H

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, cc, a-d, j-m, i, n-u (independently selected) = 0 or 1;

dd, v-z = 0.

- ↓ 1. endo-H  
2. galactosyltransferase, UDP-Gal-PEG

e-h = 1 to 4;

aa, bb, dd, a-d, i, j-u (independently selected) = 0 or 1;

cc, v-z = 0; R' = -Gal-PEG.

FIG. 42I

182/498

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, cc, a-d, j-m, i, n-u (independently selected) = 0 or 1;

dd, v-z = 0.

- ↓
1. ST3Gal3, CMP-SA
  2. endo-H
  3. galactosyltransferase, UDP-Gal-PEG

e-h = 1 to 4;

aa, bb, dd, a-d, i, j-u (independently selected) = 0 or 1;

cc, v-z = 0; R' = -Gal-PEG.

FIG. 42J

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, cc, a-d, j-m, i, n-u (independently selected) = 0 or 1;

dd, v-z = 0.

- ↓
1. mannosidases
  2. GNT 1 & 2, UDP-GlcNAc
  3. galactosyltransferase, UDP-Gal-PEG

e-h = 1 to 4;

aa, a-d, i, j-y (independently selected) = 0 or 1;

bb, cc, dd, z = 0; R = PEG.

FIG. 42K

183/498

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, cc, a-d, j-m, i, n-u (independently selected) = 0 or 1;

dd, v-z = 0.

1. mannosidases

↓ 2. GNT-1, 2, 4 & 5; UDP-GlcNAc

↓ 3. galactosyltransferase, UDP-Gal

4. ST3Gal3, CMP-SA

e-h = 1 to 4;

aa, bb, cc, a-d, i, j-q (independently selected) = 0 or 1;

dd, v-z = 0.

FIG. 42L

CHO, BHK, 293S cells, Vero, MDCK, HEKC  
expressed Factor VIII.

e-h = 1 to 4;

aa, bb, cc, a-d, j-m, i, n-u (independently selected) = 0 or 1;

dd, v-z = 0.

↓ 1. mannosidases

↓ 2. GNT-1, UDP-GlcNAc-PEG

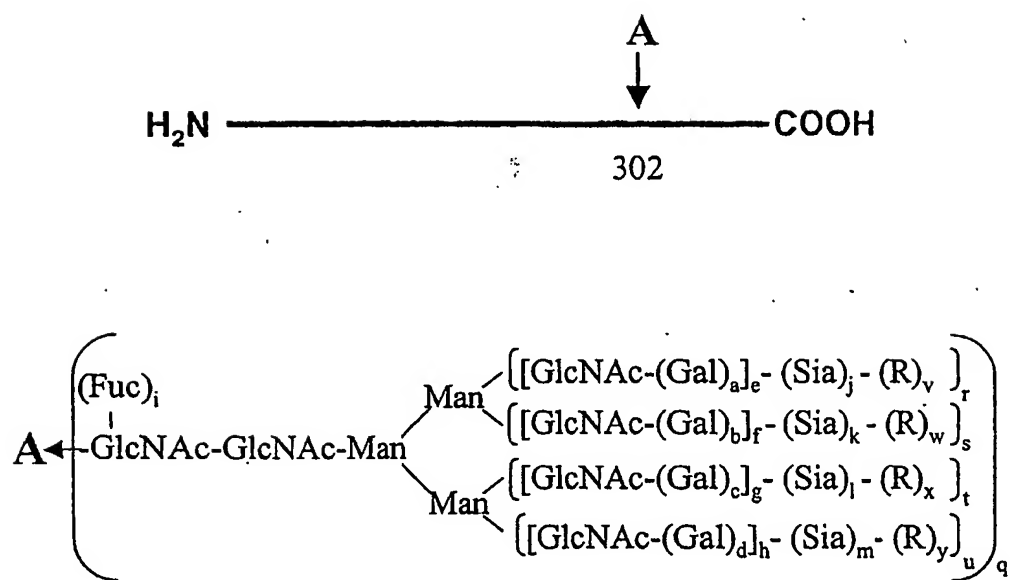
e-h = 0 to 4;

aa, a-d, i, j-y (independently selected) = 0 or 1;

bb, cc, dd, z = 0.

FIG. 42M

184/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = polymer.

FIG. 43A

185/498

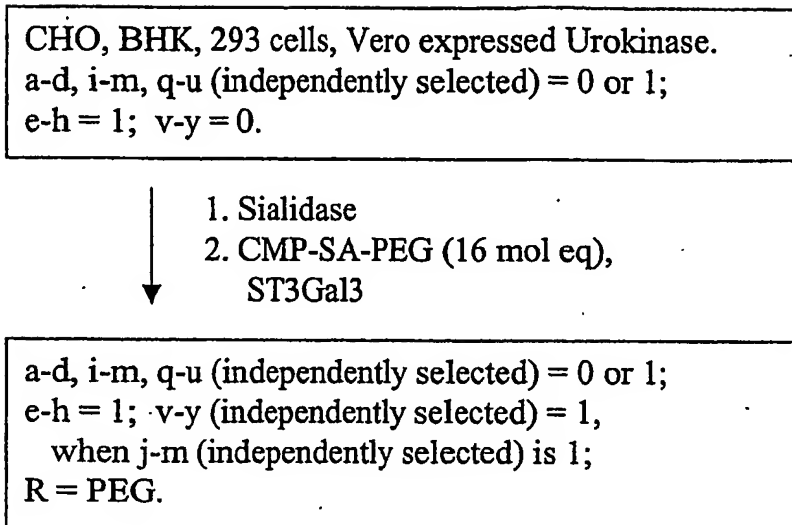


FIG. 43B

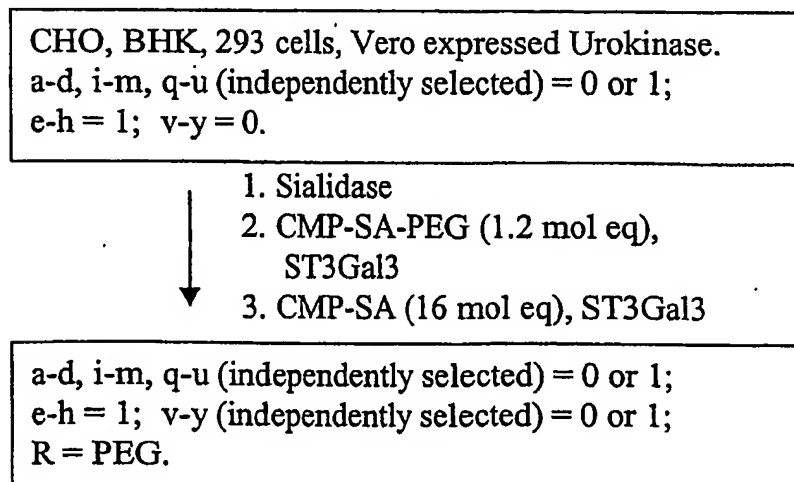


FIG. 43C



186/498

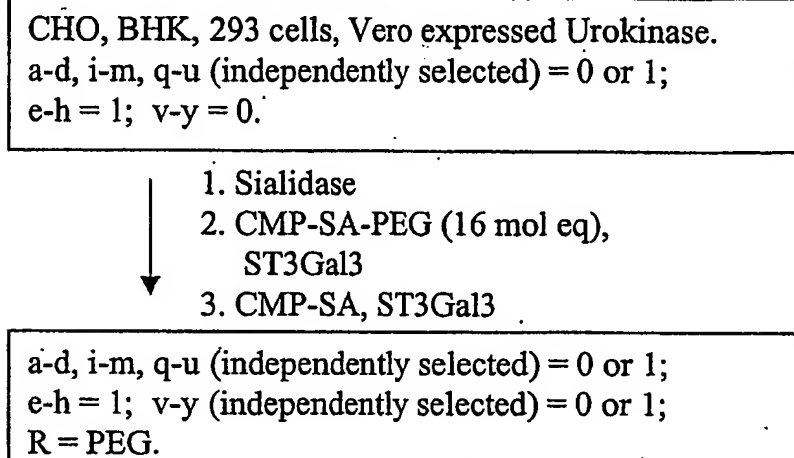


FIG. 43D

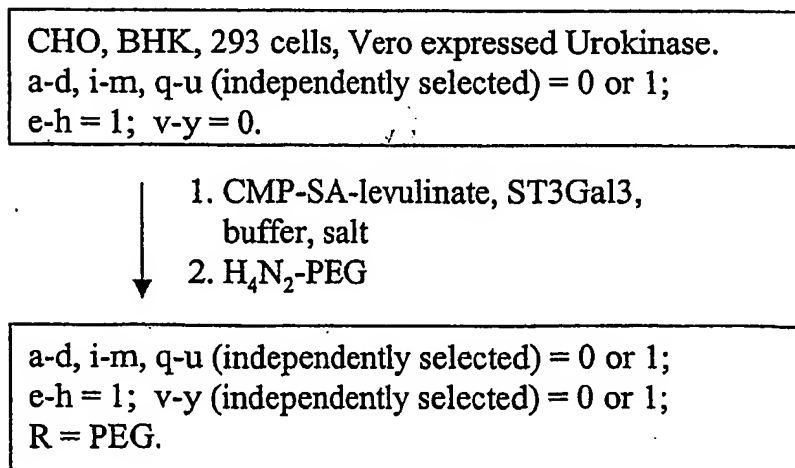


FIG. 43E

187/498

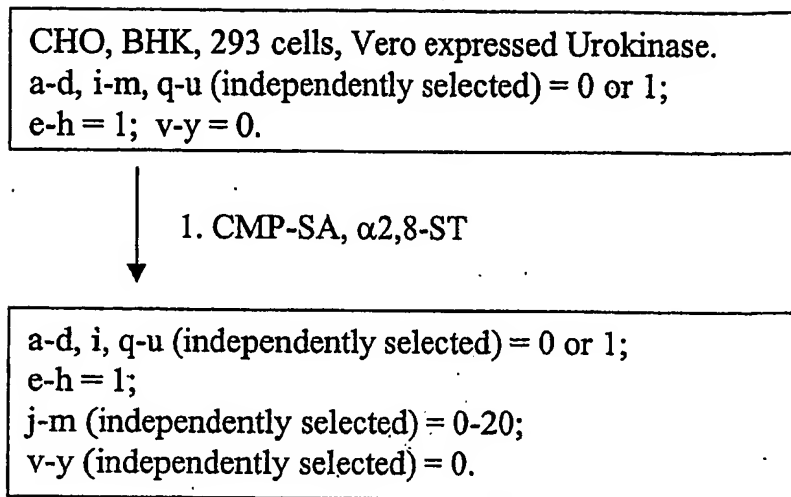
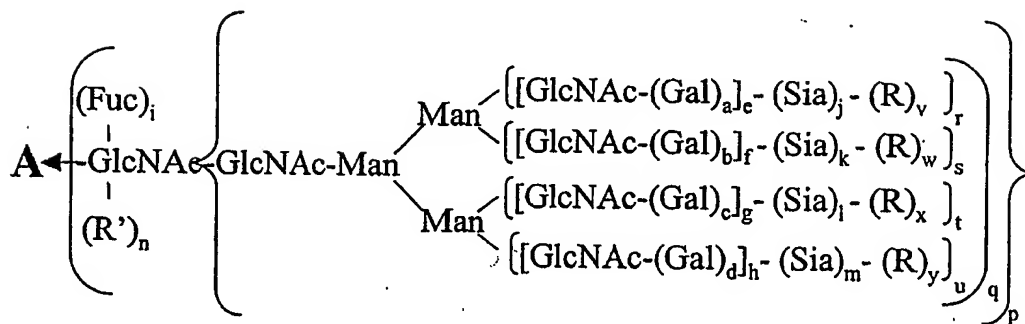
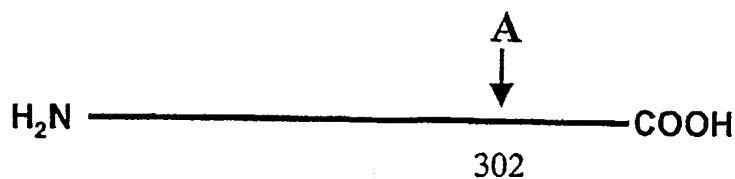


FIG. 43F

188/498



a-d, i, n, p-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0;

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group,  
glycoconjugate.

FIG. 43G

189/498

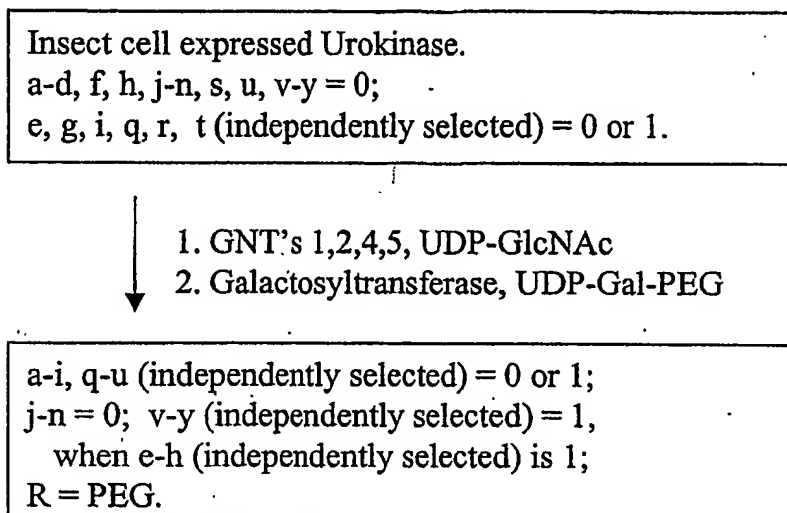


FIG. 43H

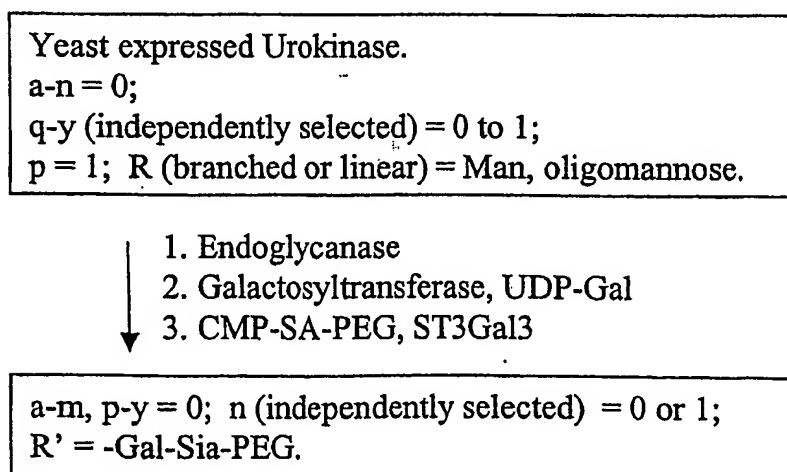


FIG. 43I

190/498

CHO, BHK, 293 cells, Vero expressed Urokinase.  
 a-d, i-m, q-u (independently selected) = 0 or 1;  
 e-h = 1; n, v-y = 0.

- ↓
1. CMP-SA-linker-SA-CMP, ST3Gal3
  2. ST3Gal1, desialylated Urokinase produced in CHO.
  - ↓ 3. CMP-SA, ST3Gal3, ST3Gal1

a-m, q-u (independently selected) = 0 or 1;  
 p = 1; n = 0;  
 v-y (independently selected) = 0 or 1;  
 R = linker-Urokinase.

FIG. 43J

Isolated Urokinase.  
 a-d, i-m, q-u (independently selected) = 0 or 1;  
 e-h = 1; v-y = 0; n = 0;  
 Sia (independently selected) = Sia or SO<sub>4</sub>;  
 Gal (independently selected) = Gal or GalNAc;  
 GlcNAc (independently selected) = GlcNAc or GlcNAc-Fuc.

- ↓
1. sulfohydrolase
  2. CMP-SA-PEG, sialyltransferase

a-d, i-m, q-u (independently selected) = 0 or 1;  
 n = 0; e-h = 1; Sia = Sia;  
 Gal (independently selected) = Gal or GalNAc;  
 GlcNAc (independently selected) = GlcNAc or GlcNAc-Fuc.  
 v-y (independently selected) = 0 or 1;  
 R = PEG.

FIG. 43K

191/498

Isolated Urokinase.

a-d, i-m, q-u (independently selected) = 0 or 1;

e-h = 1; n = 0; v-y = 0;

Sia (independently selected) = Sia or SO<sub>4</sub>;

Gal (independently selected) = Gal or GalNAc;

GlcNAc (independently selected) = GlcNAc or GlcNAc-Fuc.

↓  
1. sulfohydrolase, hexosaminidase

2. UDP-Gal-PEG, galactosyltransferase

a-d, i, q-u (independently selected) = 0 or 1;

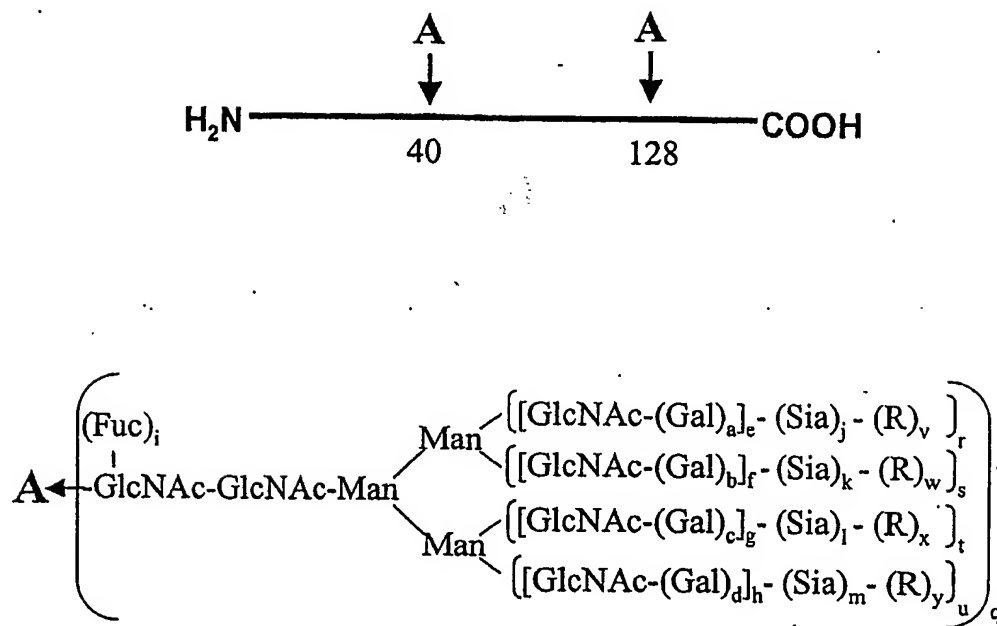
e-h = 1; j-n = 0; Gal (independently selected) = Gal;

GlcNAc (independently selected) = GlcNAc or GlcNAc-Fuc;

v-y (independently selected) = 0 or 1; R = PEG.

FIG. 43L

192/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = polymer, glycoconjugate.

FIG. 44A

193/498

CHO, BHK, 293 cells, Vero expressed DNase I.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG (16 mol eq),  
ST3Gal3

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1;  
v-y (independently selected) = 1,  
when j-m (independently selected) is 1;  
R = PEG.

FIG. 44B

CHO, BHK, 293 cells, Vero expressed DNase I.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG (1.2 mol eq), ST3Gal3
  3. CMP-SA (16 mol eq), ST3Gal3

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y (independently selected) = 0 or 1;  
R = PEG.

FIG. 44C



194/498

CHO, BHK, 293 cells, Vero expressed DNase I.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. Sialidase
  2. CMP-SA-PEG (16 mol eq), ST3Gal3
  3. CMP-SA, ST3Gal3

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y (independently selected) = 0 or 1;  
R = PEG.

FIG. 44D

CHO, BHK, 293 cells, Vero expressed DNase I.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. CMP-SA-levulinate, ST3Gal3,  
buffer, salt
  2. H<sub>4</sub>N<sub>2</sub>-PEG

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y (independently selected) = 0 or 1;  
R = PEG.

FIG. 44E

195/498

CHO, BHK, 293 cells, Vero expressed DNase I.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

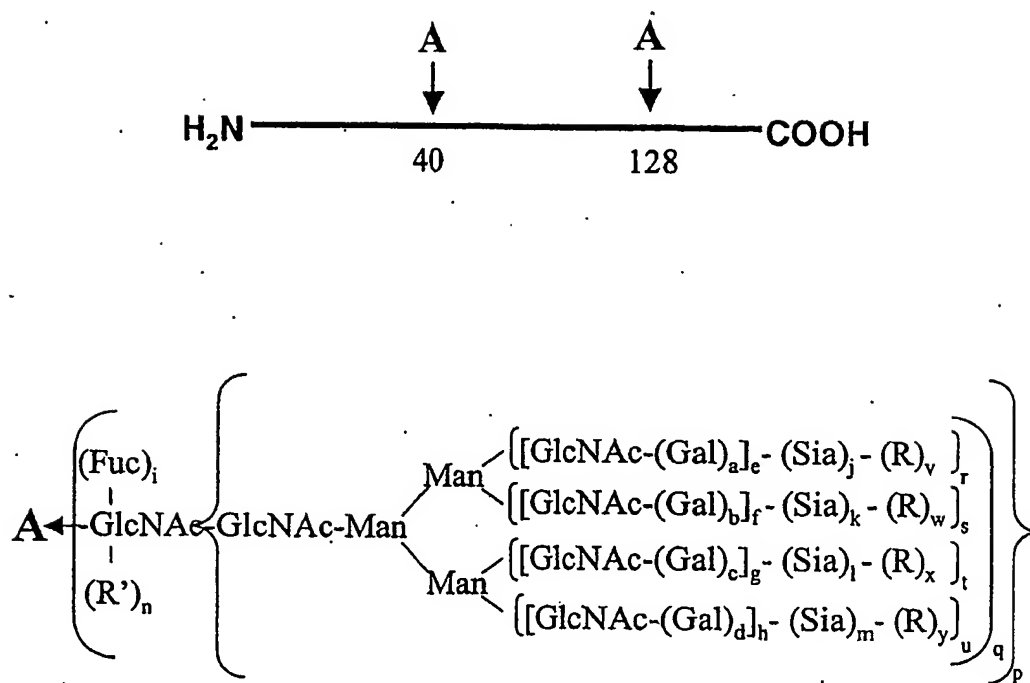


1. CMP-SA,  $\alpha$ 2,8-ST

a-d, i, q-u (independently selected) = 0 or 1;  
e-h = 1;  
j-m (independently selected) = 0-20;  
v-y (independently selected) = 0.

FIG. 44F

196/498



a-d, i, n, p-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0;

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 44G

197/498

Insect cell expressed DNase I.  
 a-d, f, h, j-n, s, u, v-y = 0;  
 e, g, i, q, r, t (independently selected) = 0 or 1.

- ↓
1. GNT's 1,2,4,5, UDP-GlcNAc
  2. Galactosyltransferase, UDP-Gal-PEG

a-i, q-u (independently selected) = 0 or 1; j-n = 0;  
 v-y (independently selected) = 1,  
 when e-h (independently selected) is 1;  
 R = PEG.

FIG. 44H

Yeast expressed DNase I.  
 a-n = 0;  
 q-y (independently selected) = 0 to 1;  
 p = 1; R (branched or linear) = Man, oligomannose.

- ↓
1. Endoglycanase
  2. Galactosyltransferase, UDP-Gal
  3. CMP-SA-PEG, ST3Gal3

a-n, p-y = 0; n (independently selected) = 0 or 1;  
 R' = -Gal-Sia-PEG.

FIG. 44I

198/498

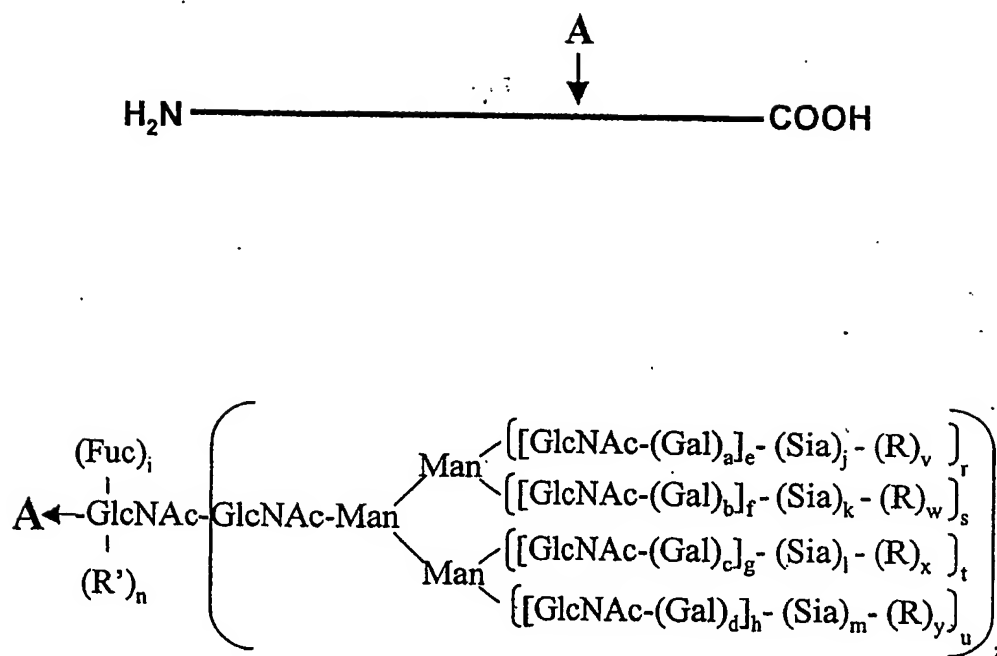
CHO, BHK, 293 cells, Vero expressed DNase I.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; n, v-y = 0.

- ↓
1. CMP-SA-linker-SA-CMP, ST3Gal3
  2. ST3Gal1, desialylated alpha-1-Proteinase inhibitor.
  3. CMP-SA, ST3Gal3, ST3Gal1

a-m, q-u (independently selected) = 0 or 1;  
p = 1; n = 0;  
v-y (independently selected) = 0 or 1;  
R = linker- alpha-1-Proteinase inhibitor.

FIG. 44J

199/498



a-d, i, r-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

$j$ -m (independently selected) = 0 or 1.

$$n, v-y = 0; z = 0 \text{ or } 1;$$

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group,

glycoconjugate.

**FIG. 45A**

200/498

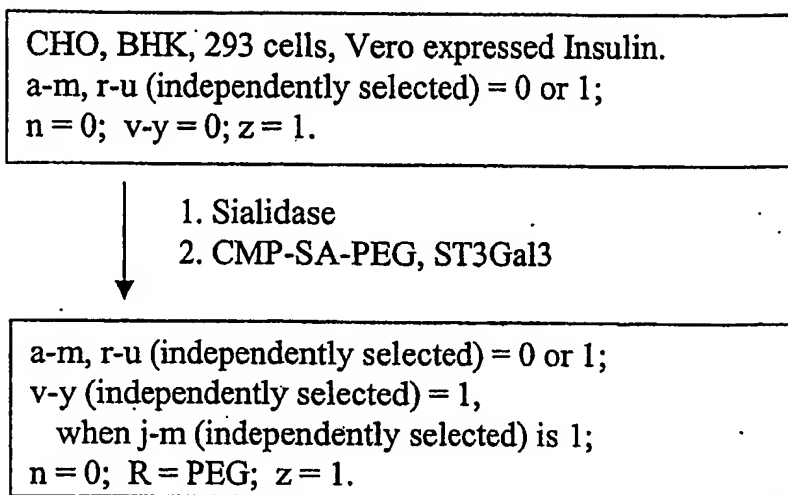


FIG. 45B

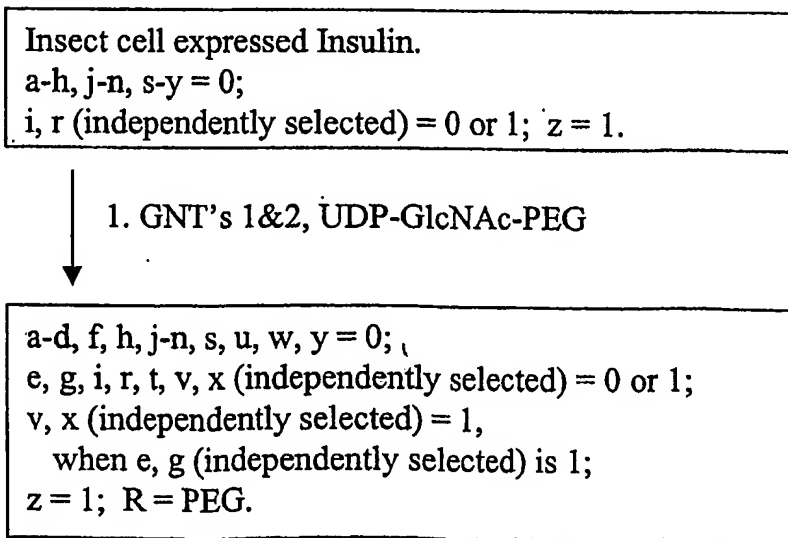


FIG. 45C

201/498

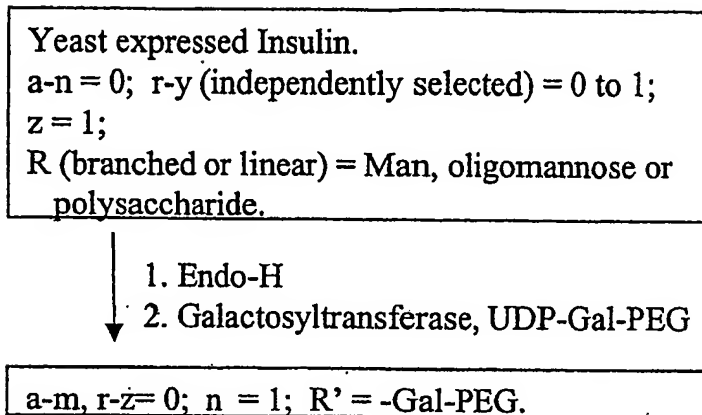
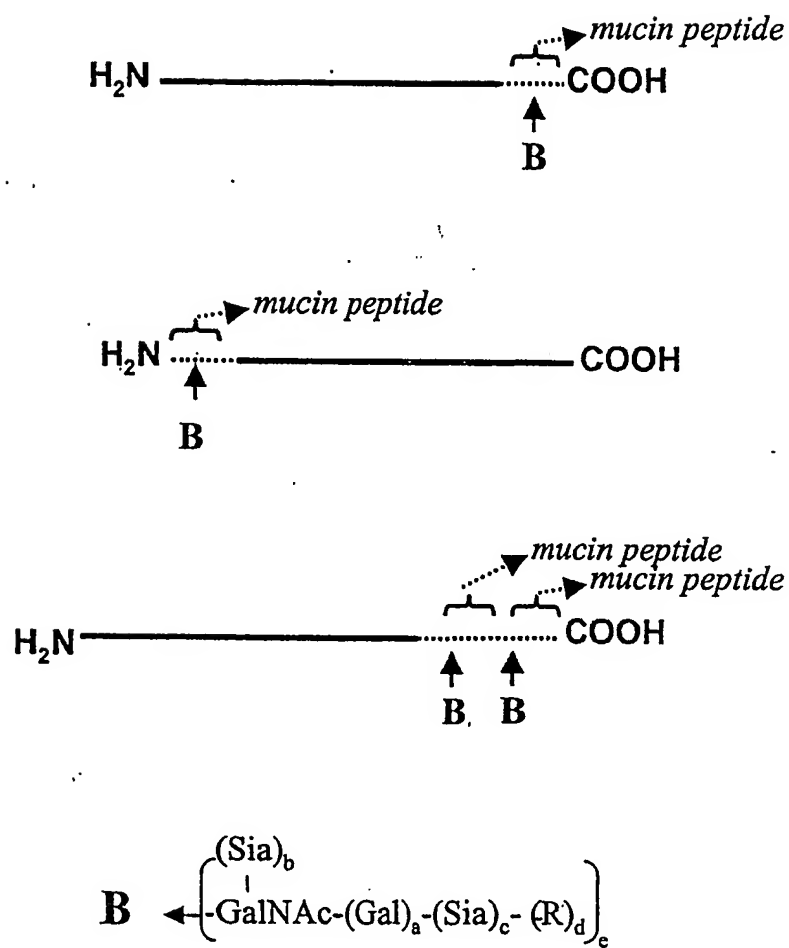


FIG. 45D



202/498



a-c, e (independently selected) = 0 or 1;  
d = 0; R = polymer

FIG. 45E

203/498

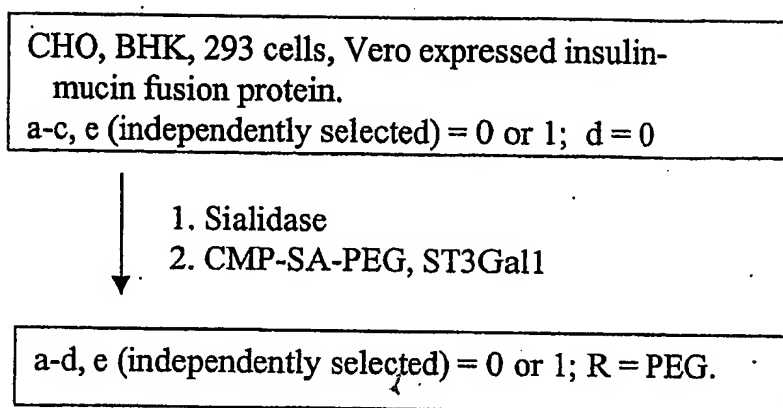


FIG. 45F

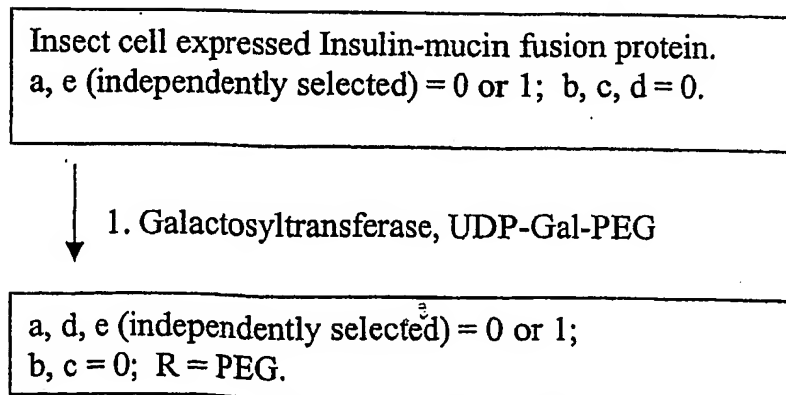


FIG. 45G

204/498

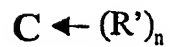
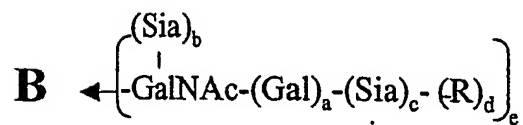
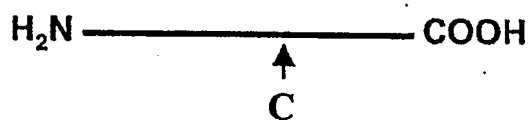
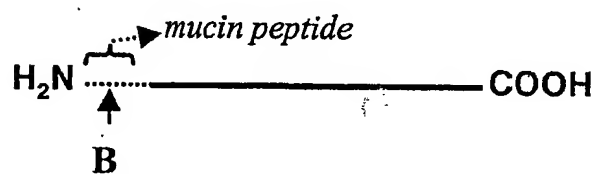
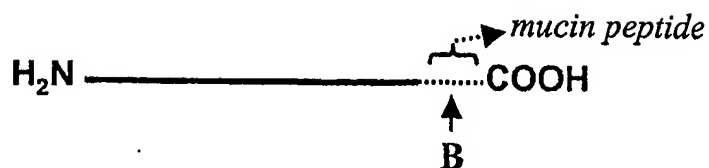
E. coli expressed Insulin-mucin fusion protein.  
a-e = 0.

- ↓
1. GalNAc Transferase, UDP-GalNAc
  2. CMP-SA-PEG, sialyltransferase

c, d, e (independently selected) = 0 or 1;  
a, b = 0; R = PEG.

FIG. 45H

205/498



a-c, e (independently selected) = 0 or 1;  
 d = 0; R = modifying group, mannose,  
 oligo-mannose.

FIG. 45I

206/498

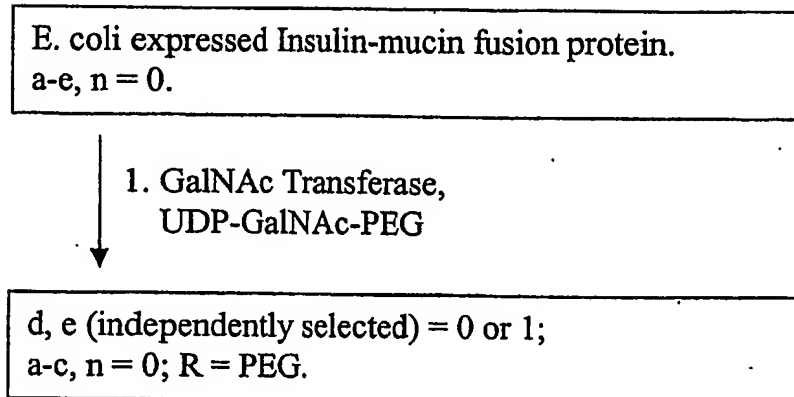


FIG. 45J

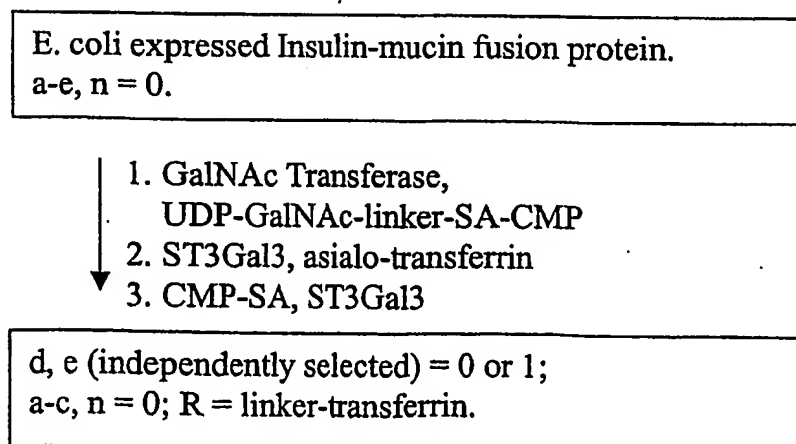


FIG. 45K

207/498

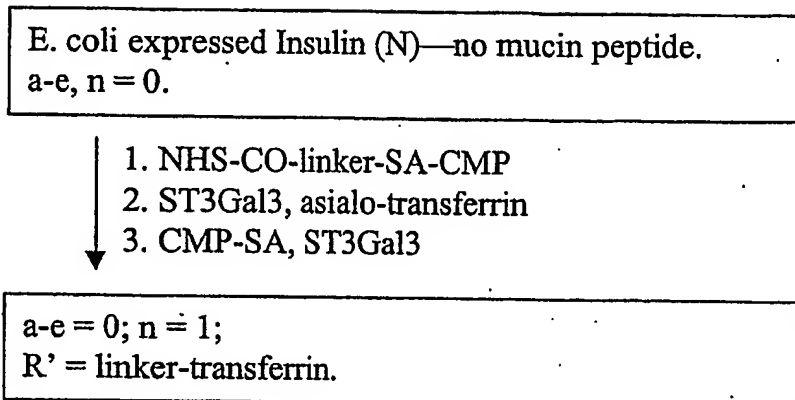
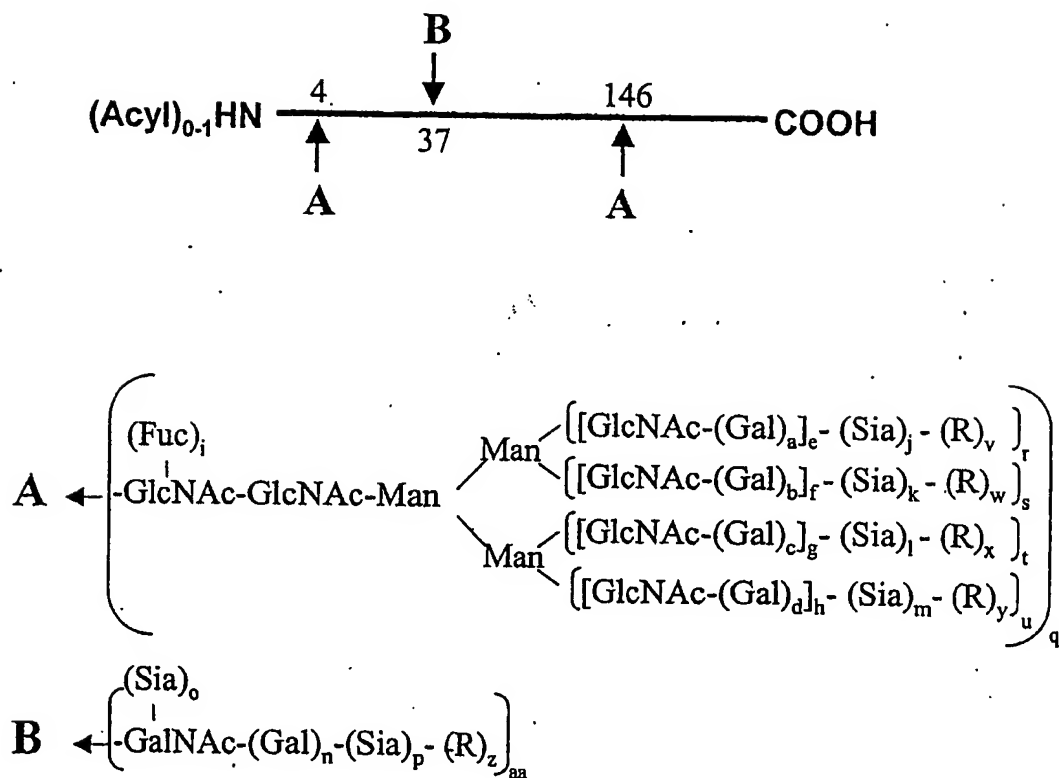


FIG. 45L

208/498



a-d, i, n-u, aa (independently selected) = 0 or 1.  
 e-h (independently selected) = 0 to 6.  
 j-m (independently selected) = 0 to 100.  
 v-y = 0; R = polymer, glycoconjugate.

FIG. 46A

209/498

CHO, BHK, 293 cells, Vero expressed M-antigen.  
 a-d, i-m, o-u, aa (independently selected) = 0 or 1;  
 n, e-h = 1; v-z = 0.

- ↓
1. Sialidase
  2. CMP-SA-linker-lipid-A,  
ST3Gal3

a-d, i-m, q-u, aa (independently selected) = 0 or 1;  
 o, p, z = 0; n, e-h = 1;  
 v-y (independently selected) = 1,  
 when j-m (independently selected) is 1;  
 R = linker-lipid-A.

FIG. 46B

CHO, BHK, 293 cells, Vero expressed M-antigen.  
 a-d, i-m, o-u, aa (independently selected) = 0 or 1;  
 n, e-h = 1; v-z = 0.

- ↓
1. sialidase
  2. CMP-SA-linker-tetanus toxin, ST3Gal1
  3. CMP-SA, ST3Gal3

a-d, i-m, p-u, z, aa (independently selected) = 0 or 1;  
 o, v-y = 0; n, e-h = 1; R = tetanus toxin.

FIG. 46C



210/498

NSO expressed M-antigen.

a-d, i-n, o-u, aa (independently selected) = 0 or 1;

e-h = 1; v-z = 0;

Sia (independently selected) = Sia or Gal.

- ↓
1.  $\alpha$ -galactosidase
  2. CMP-SA, ST3Gal3
  2. CMP-SA-KLH, ST3Gal1

a-d, i-n, p-u, z, aa (independently selected) = 0 or 1;

e-h = 1; o, v-y = 0;

z = 1, when p = 1;

R = KLH.

FIG. 46D

Yeast expressed M-antigen.

a-p, z = 0; q-y, aa (independently selected) = 0 to 1;

R (branched or linear) = Man, oligomannose;

GalNAc = Man.

- ↓
1.  $\alpha$ 1,2-mannosidase
  2. GNT 1,
- UDP-GlcNAc-linker-diphtheria toxin.

e, q, l, m, r, t, u, v, aa (independently selected) = 0 or 1;

a-d, f-h, j, k, n-p, s, w-z = 0;

Sia = Man; R = linker-diphtheria toxin.

FIG. 46E

211/498

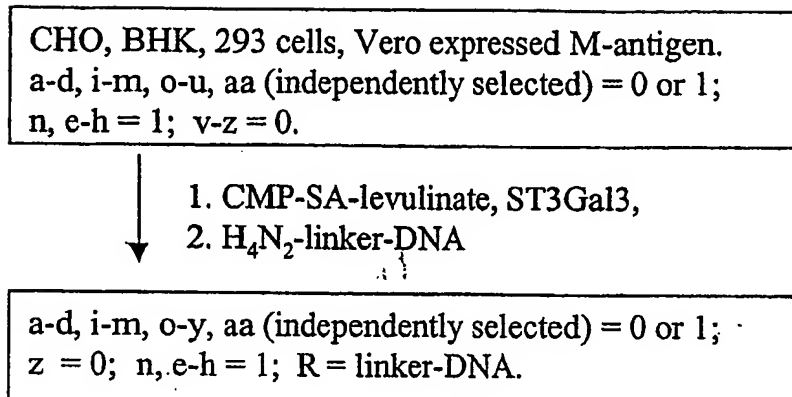


FIG. 46F

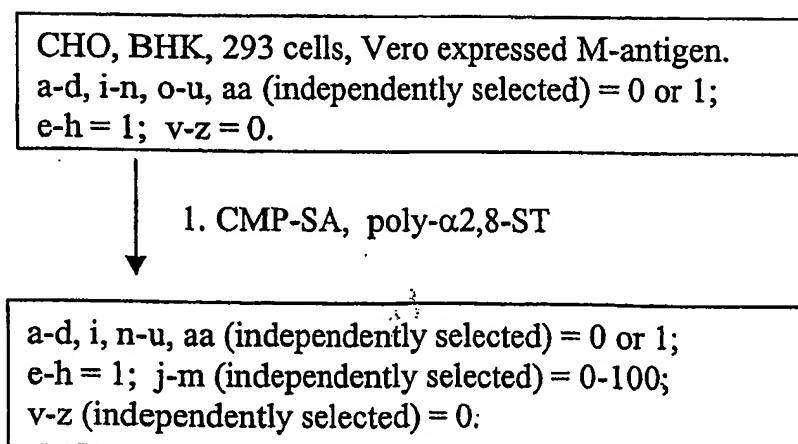
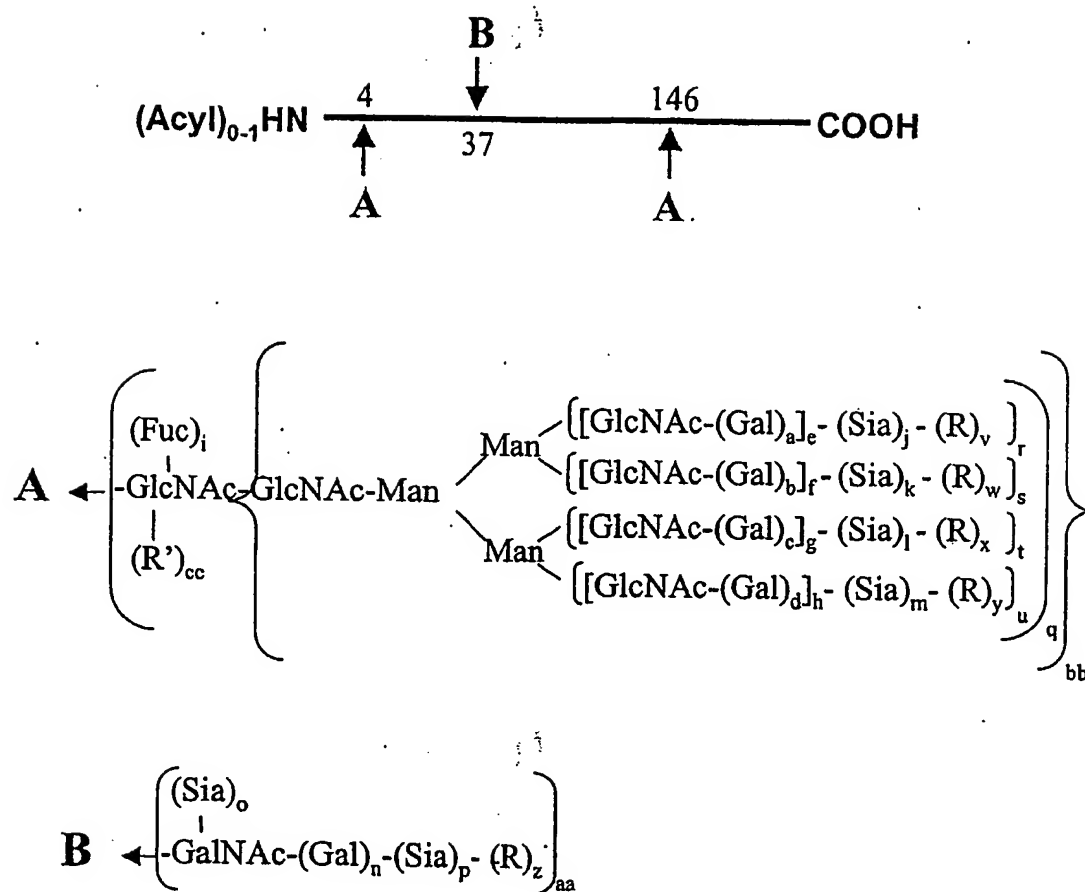


FIG. 46G

212/498



a-d, i, n, q-u, aa, bb, (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-p (independently selected) = 0 to 100.

$$Cc, v-y = 0;$$

R = modifying group, mannose, oligo-mannose.

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 46H

213/498

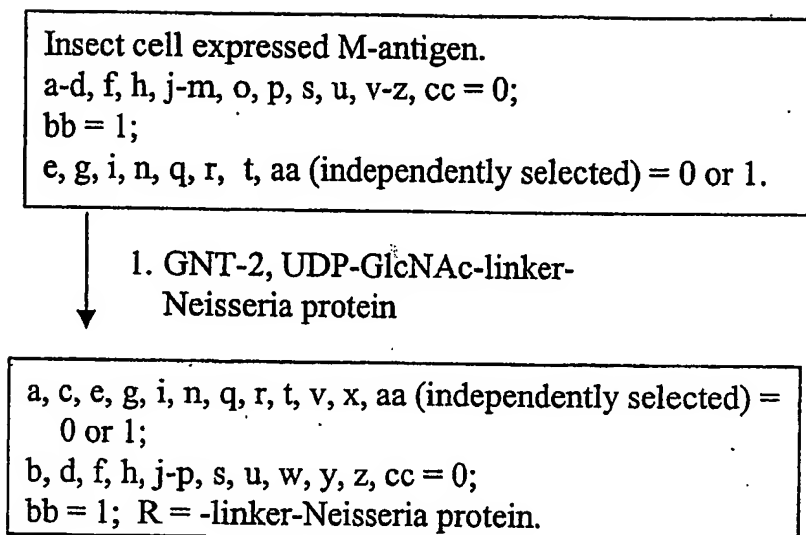


FIG. 46I

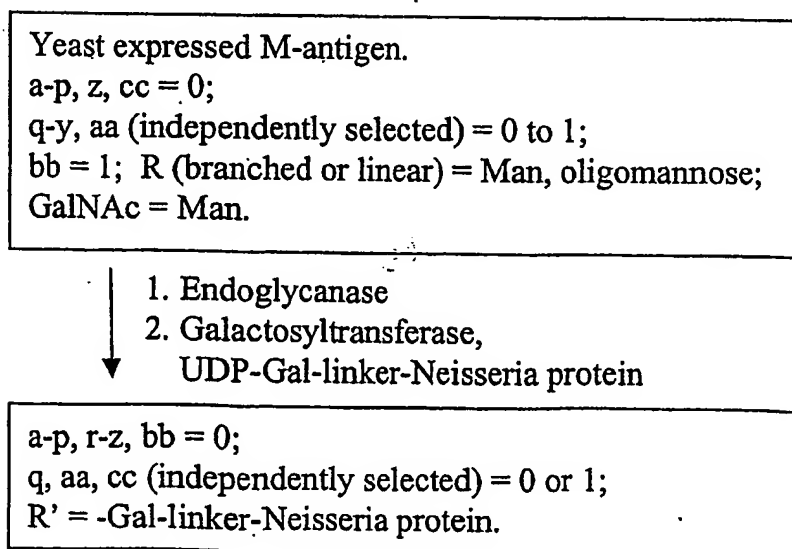


FIG. 46J

214/498


Yeast expressed M-antigen.

a-p, z, cc = 0;

q-y, aa (independently selected) = 0 to 1; bb = 1;

R (branched or linear) = Man, oligomannose;

GalNAc = Man.

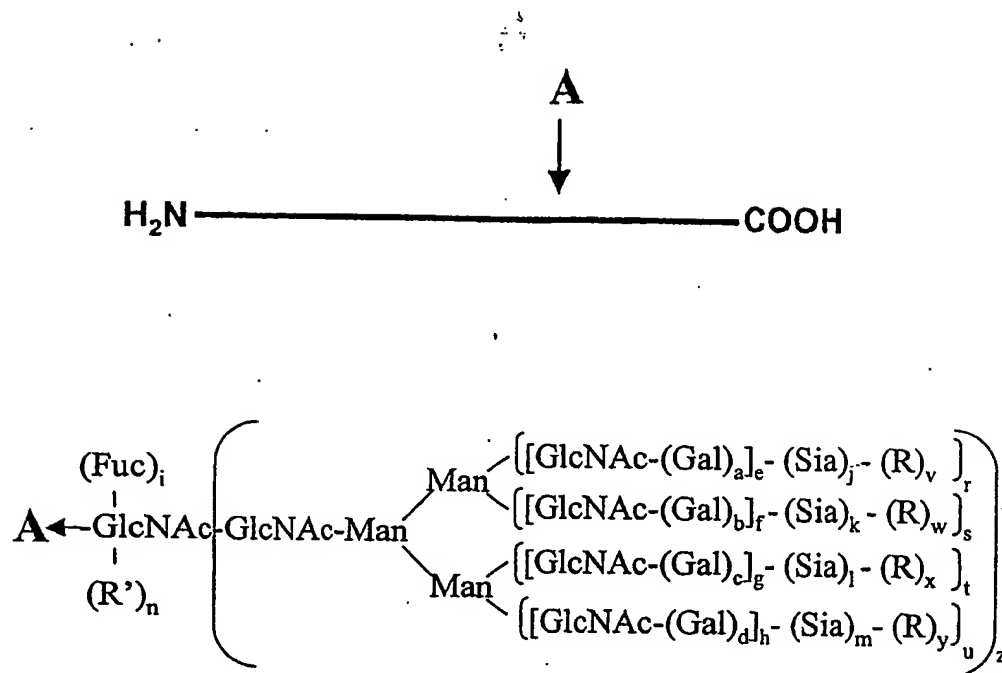
- 
1. mannosidases
  2. GNT 1 & 2, UDP-GlcNAc
  3. UDP-Gal, Galactosyltransferase,
  4. CMP-SA, sialyltransferase

a, c, e, g, j, l, q, r, t, aa (independently selected) = 0 or 1;

b, d, f, h, k, m-p, s, u-z, cc = 0; bb = 1.

FIG. 46K

215/498



a-d, i, r-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 or 1.

n, v-y = 0; z = 0 or 1;

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group,  
glycoconjugate.

FIG. 47A

216/498

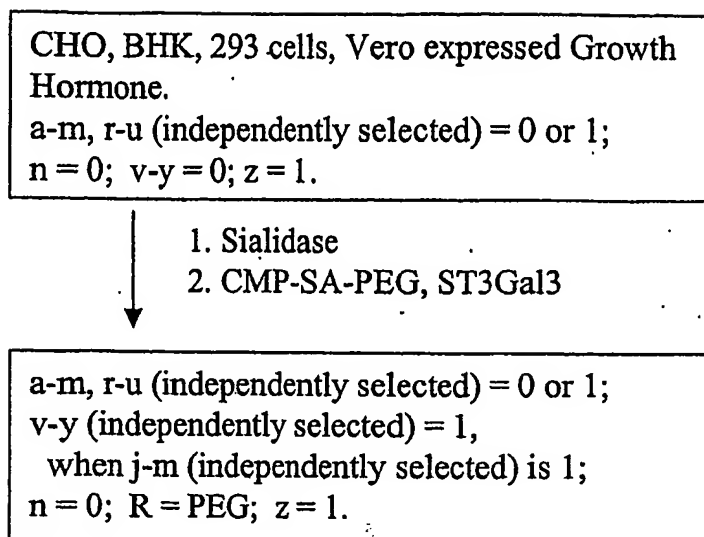


FIG. 47B

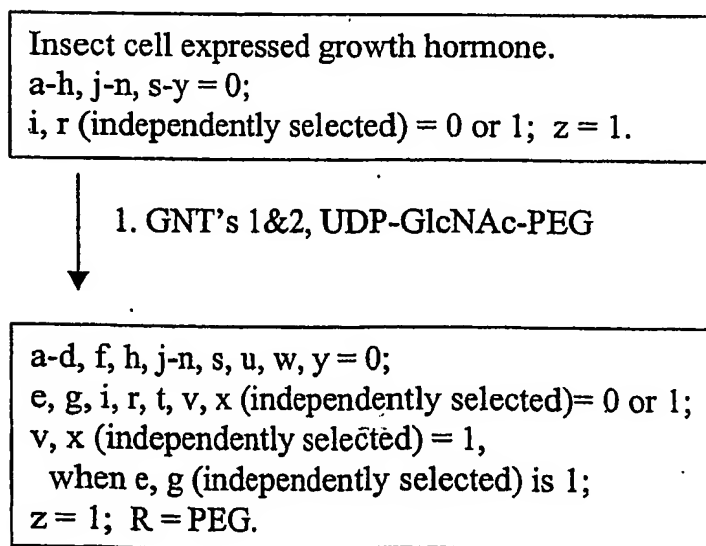


FIG. 47C

217/498

Yeast expressed growth hormone.  
a-n = 0; r-y (independently selected) = 0 to 1;  
z = 1;  
R (branched or linear) = Man, oligomannose or  
polysaccharide.

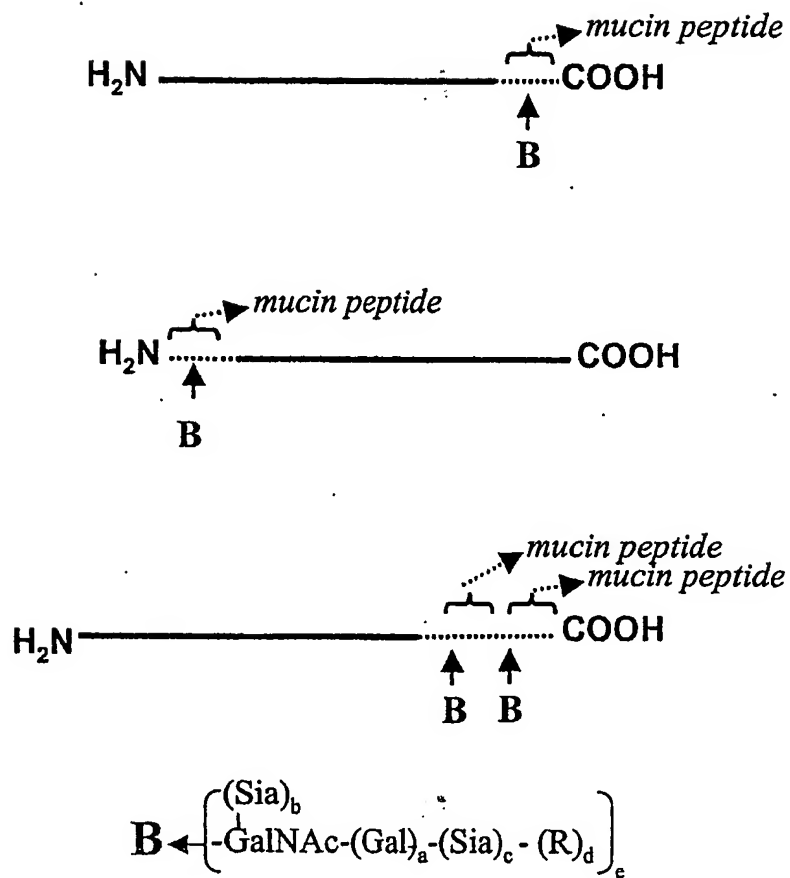
- ↓  
1. Endo-H  
2. Galactosyltransferase, UDP-Gal-PEG

a-m, r-z= 0; n = 1; R' = -Gal-PEG.

FIG. 47D



218/498



a-c, e (independently selected) = 0 or 1;  
 d = 0;  
 R = modifying group, mannose, oligo-  
 mannose.

FIG. 47E

219/498

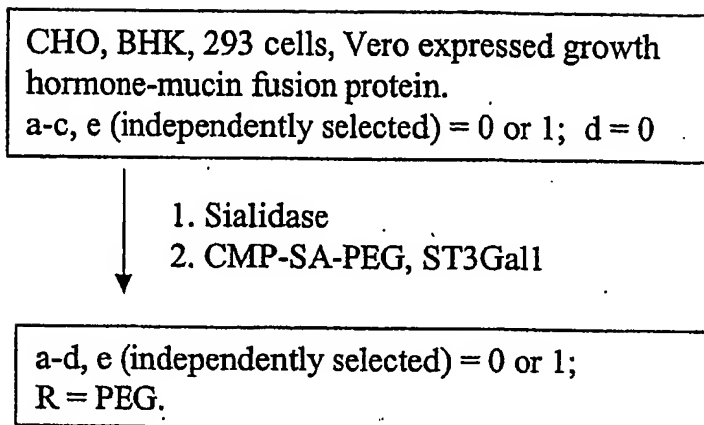


FIG. 47F

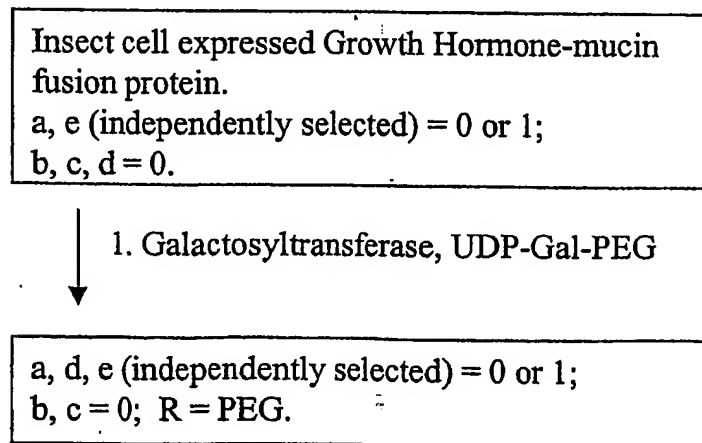


FIG. 47G

220/498

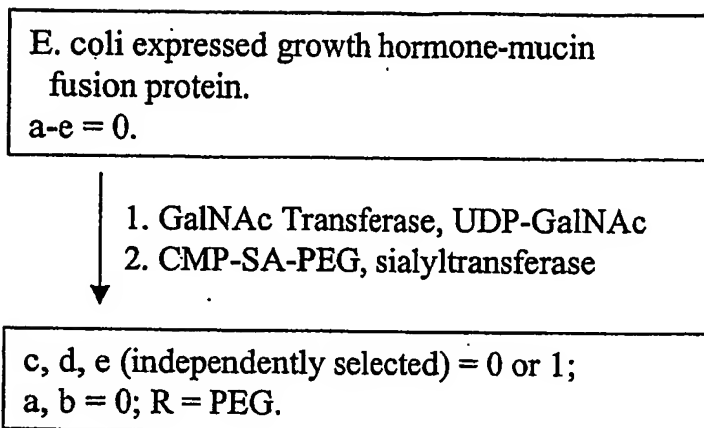


FIG. 47H

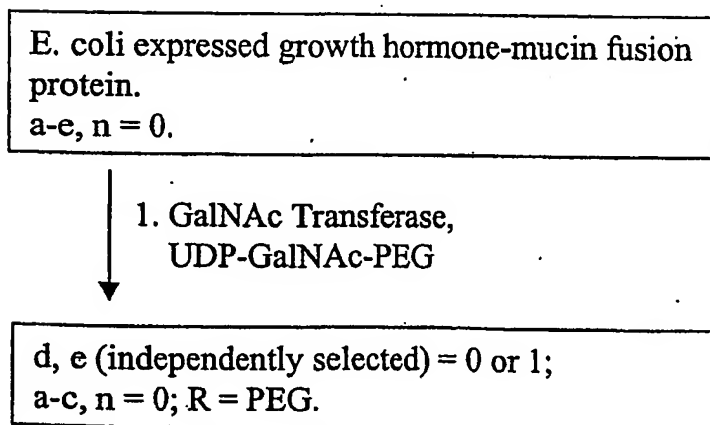


FIG. 47I

221/498

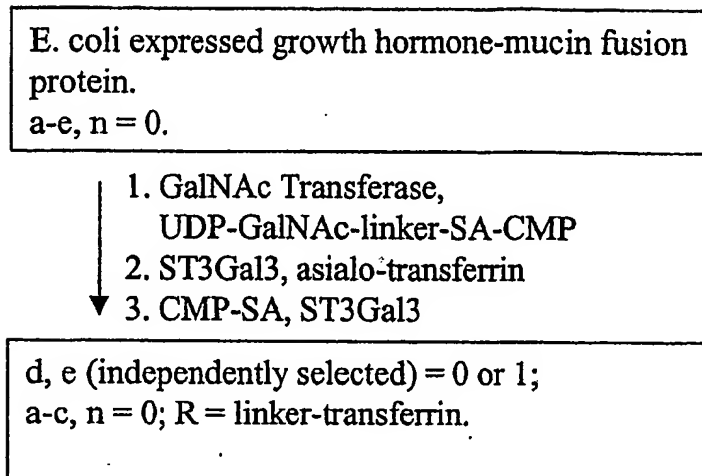


FIG. 47J

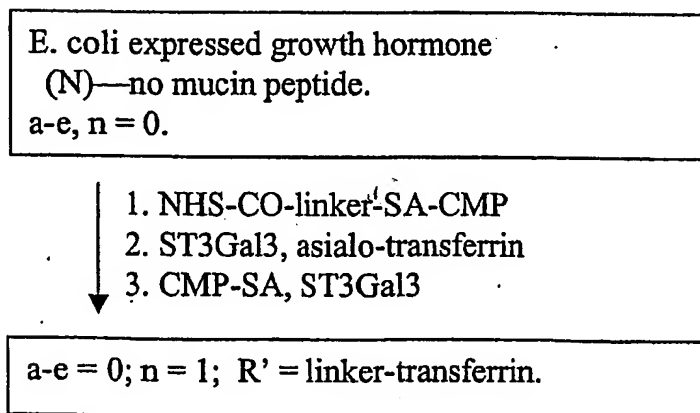
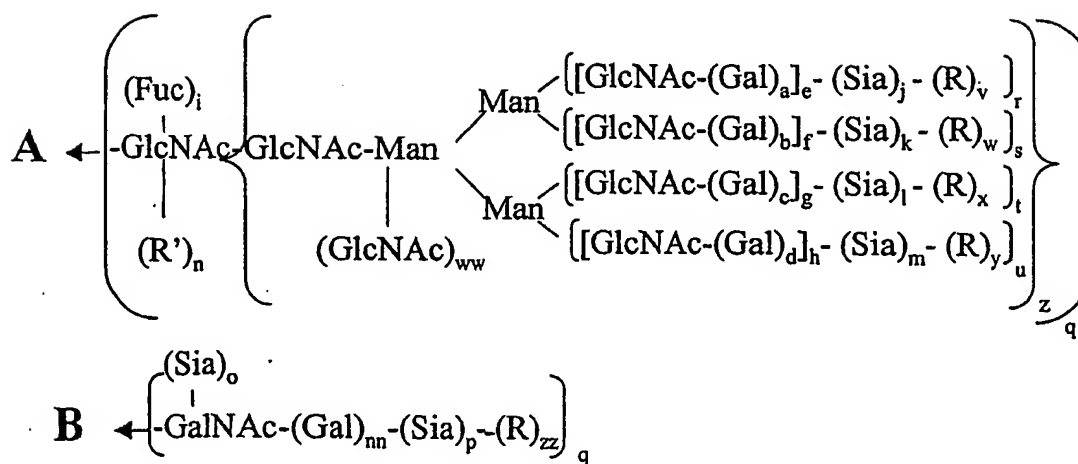
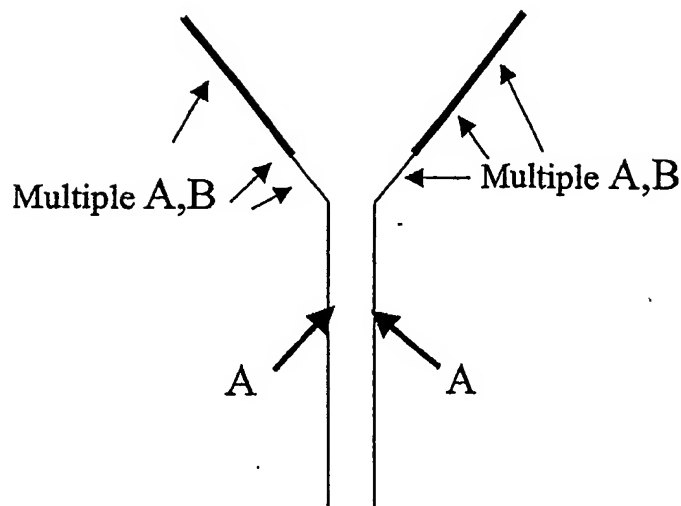


FIG. 47K

222/498



a-d, i-m, q-u, w, z, nn, ww, zz (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

n, v-y = 0;

R = modifying group, mannose; oligo-mannose;

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 48A

223/498

CHO, BHK, 293 cells, Vero or transgenic animals  
expressed TNF Receptor IgG Fusion.

a-m, o-u, aa (independently selected) = 0 or 1;  
n = 1; v-z = 0.

- ↓
1. CMP-SA, ST3Gal1
  2. galactosyltransferase, UPD-Gal
  3. CMP-SA-PEG, ST3Gal3

a-m, o-u, v-y, aa (independently selected) = 0 or 1;  
n = 1; z = 0; R = PEG.

FIG. 48B

CHO, BHK, 293 cells, Vero expressed  
TNF Receptor IgG Fusion.

a-m, o-u, aa (independently selected) = 0 or 1;  
n = 1; v-z = 0.

- ↓
1. sialidase
  2. CMP-SA-PEG, ST3Gal1

a-i, p-u, z, aa (independently selected) = 0 or 1;  
n = 1; o, j-m, v-y = 0; R = PEG.

FIG. 48C

224/498

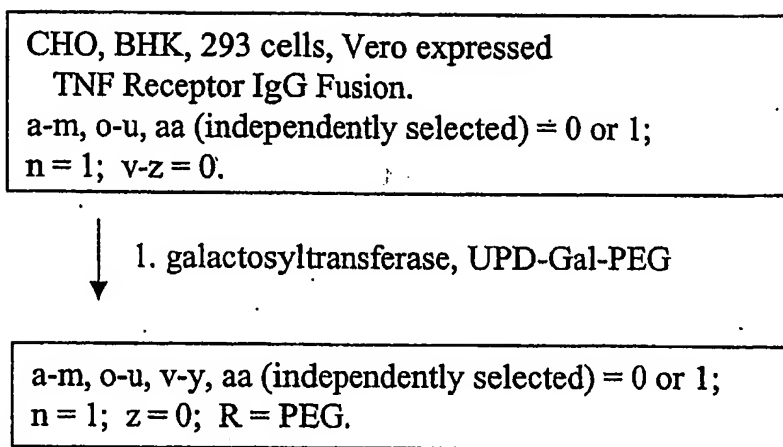


FIG. 48D

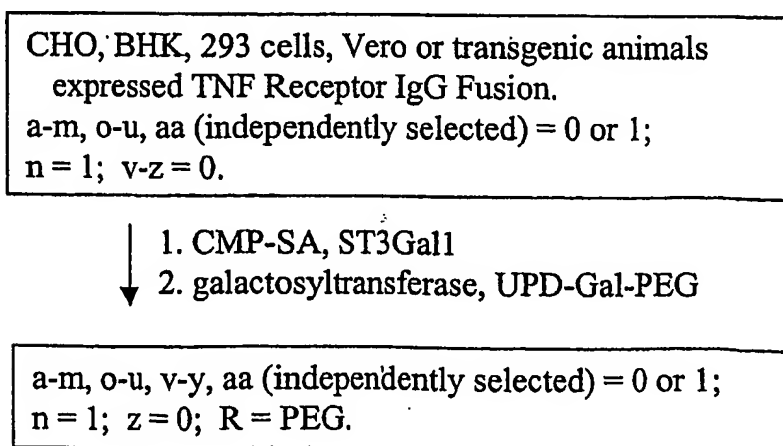


FIG. 48E

225/498

CHO, BHK, 293 cells, Vero or transgenic animals  
expressed TNF Receptor IgG Fusion.  
a-m, o-u, aa (independently selected) = 0 or 1;  
n = 1; v-z = 0.

↓ 1. CMP-SA-levulinate, ST3Gal1  
2. H<sub>4</sub>N<sub>2</sub>-PEG

a-m, o-u, v-y, aa (independently selected) = 0 or 1;  
n = 1; z = 0; R = PEG.

FIG. 48F

CHO, BHK, 293 cells, Vero expressed  
TNF Receptor IgG Fusion.  
a-m, o-u, aa (independently selected) = 0 or 1;  
n = 1; v-z = 0.

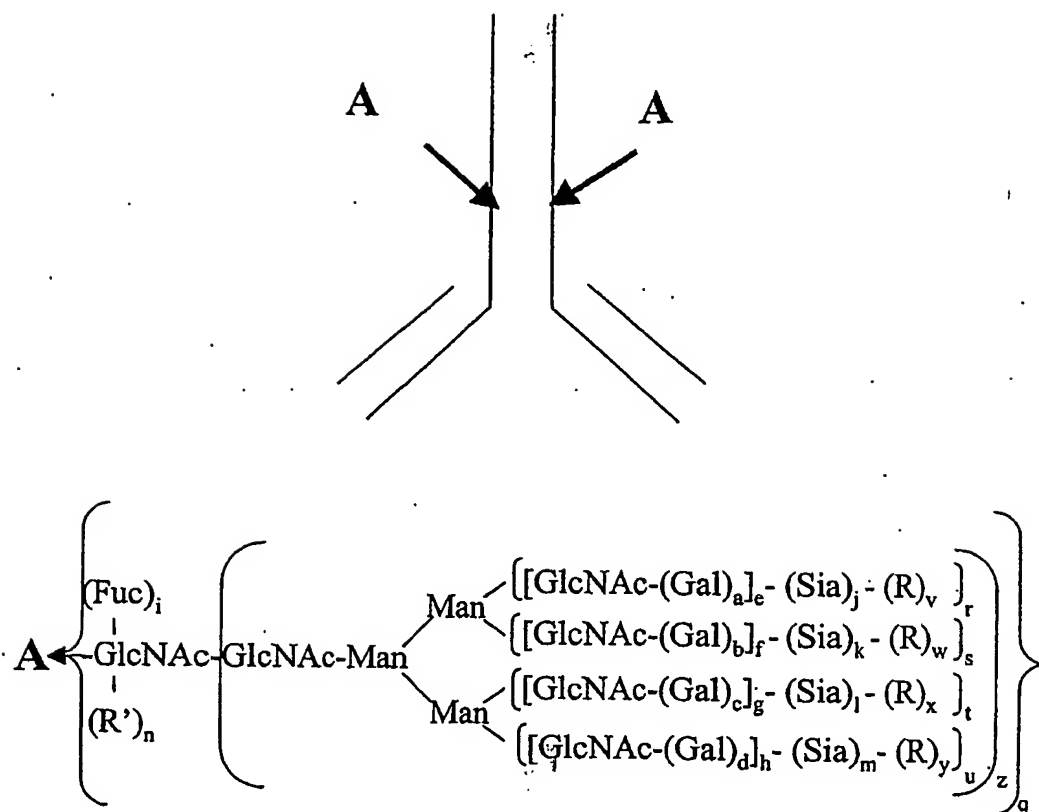
↓ 1. CMP-SA-PEG;  $\alpha$ 2,8-ST

a-i, o, q-u, v-z, aa (independently selected) = 0 or 1;  
n = 1; j-m, p (independently selected) = 0 to 2;  
v-z (independently selected) = 1,  
when j-m, p (independently selected) is 2;  
R = PEG.

FIG. 48G



226/498



a-d, i, l, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-k (independently selected) = 0 or 1.

M = 0 to 20.

n, v-y = 0; z = 0 or 1;

R = polymer, toxin, radioisotope-complex, drug, mannose, oligo-mannose.

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 49A

227/498

CHO, BHK, 293 cells, Vero expressed Herceptin.  
 a, c, i (independently selected) = 0 or 1;  
 e, g, r, t = 1; b, d, f, h, j-m, n, s, u-y = 0;  
 q, z = 1.



1. galactosyltransferase, UPD-Gal
2. CMP-SA-toxin, ST3Gal3

a, c, i, j, l (independently selected) = 0 or 1;  
 e, g, r, t = 1; R = toxin;  
 f, h, k, m, n, s, u-y = 0; q, z = 1;  
 v-y (independently selected) = 51,  
 when j, l (independently selected) is 1.

FIG. 49B

CHO, BHK, 293 cells, Vero or fungal expressed Herceptin.  
 a, c, i (independently selected) = 0 or 1;  
 e, g, r, t = 1; b, d, f, h, j-m, n, s, u-y = 0;  
 q, z = 1.



1. galactosyltransferase,  
UPD-Gal-Toxin

a, c, i (independently selected) = 0 or 1;  
 e, g, r, t = 1; f, h, j-m, n, s, u-y = 0;  
 q, z = 1; v-y (independently selected) = 1,  
 when a, c (independently selected) is 1;  
 R = toxin.

FIG. 49C

228/498

Fungi expressed Herceptin.

e, g, i, r, t (independently selected) = 0 or 1;

a-d, f, h, j-m, n, s, u-y = 0; q, z = 1.

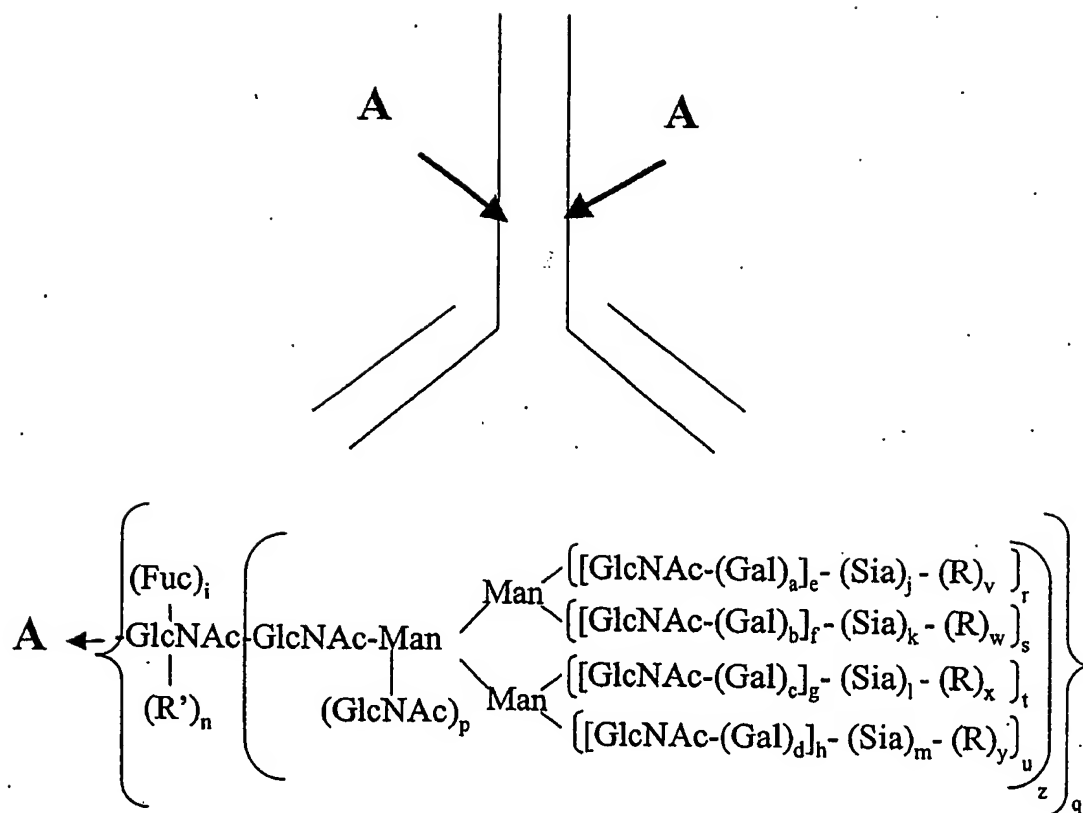
- ↓
1. Endo-H
  2. Galactosyltransferase, UDP-Gal
  - ↓ 3. CMP-SA-radioisotope complex, ST3Gal3

a-m, r-z = 0; q, n = 1;

R' = -Gal-Sia-radioisotope complex.

FIG. 49D

229/498



a-d, i, p-u, (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 or 1.

n, v-y = 0; z = 0 or 1;

R = polymer, toxin, radioisotope-complex, drug, mannose, oligo-mannose.

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 50A

230/498

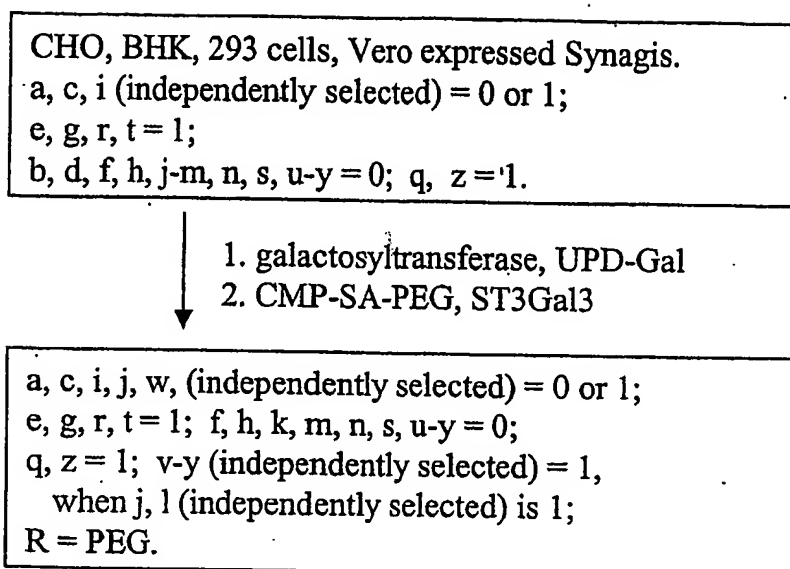


FIG. 50B

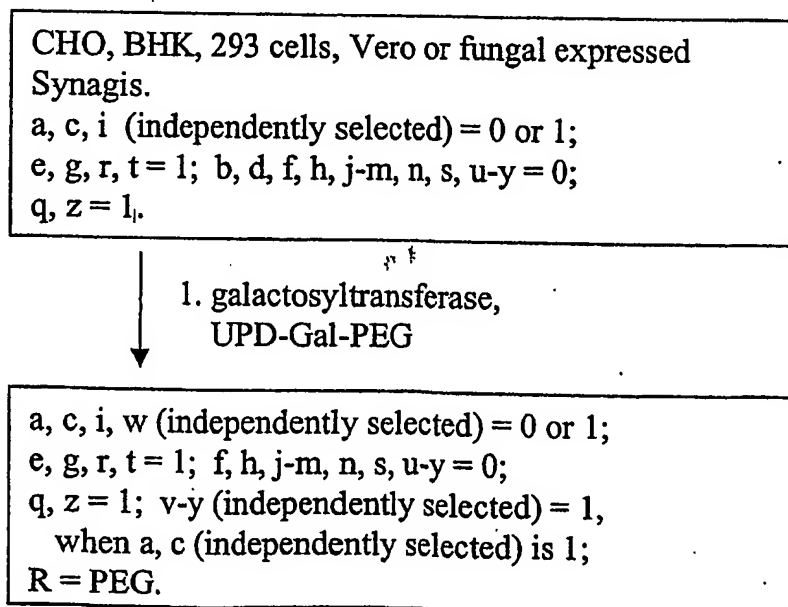


FIG. 50C

231/498

Fungi expressed Synagis.

e, g, i, r, t (independently selected) = 0 or 1;

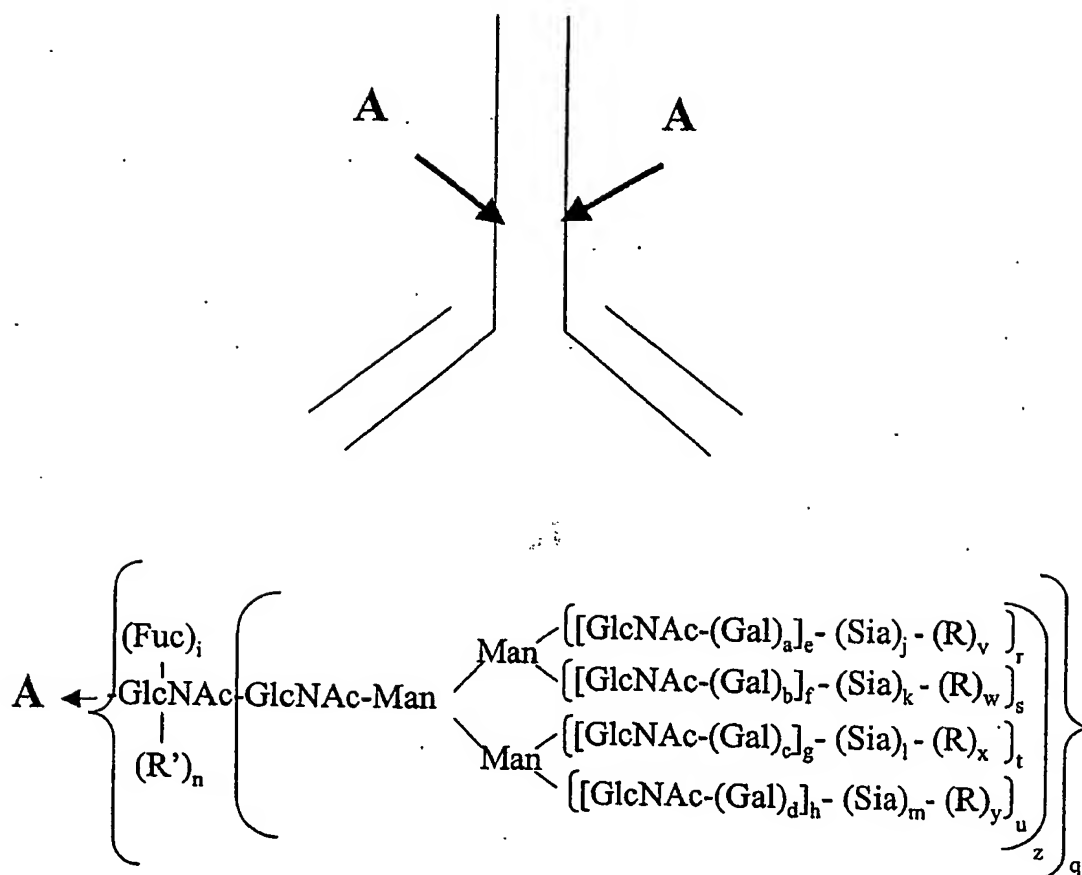
a-d, f, h, j-m, n, s, u-y = 0; q, z = 1.

- ↓
1. Endo-H
  2. Galactosyltransferase, UDP-Gal
  - 3.. CMP-SA-PEG, ST3Gal3

a-m, r-z= 0; q, n = 1; R' = -Gal-Sia-PEG.

FIG. 50D

232/498



a-d, i, q-u, w (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 20.

$$n, v-y=0; z=0 \text{ or } 1;$$

R = polymer, toxin, radioisotope-complex, drug, mannose, oligo-mannose.

**R' = H, glycosyl residue, modifying group, glycoconjugate.**

FIG. 51A

233/498

CHO, BHK, 293 cells, Vero expressed Remicade.  
 a, c, i (independently selected) = 0 or 1;  
 e, g, r, t = 1; b, d, f, h, j-m, n, s, u-y = 0;  
 q, z = 1.



1. galactosyltransferase, UPD-Gal
2. CMP-SA-PEG, ST3Gal3

a, c, i, j, l (independently selected) = 0 or 1;  
 e, g, r, t = 1; f, h, k, m, n, s, u-y = 0;  
 q, z = 1; v-y (independently selected) = 1,  
 when j, l (independently selected) is 1;  
 R = PEG.

FIG. 51B

CHO, BHK, 293 cells, Vero or fungal expressed Remicade.  
 a, c, i (independently selected) = 0 or 1;  
 e, g, r, t = 1; b, d, f, h, j-m, n, s, u-y = 0;  
 q, z = 1.



1. galactosyltransferase,  
UPD-Gal-PEG

a, c, i (independently selected) = 0 or 1;  
 e, g, r, t = 1; f, h, j-m, n, s, u-y = 0;  
 q, z = 1; v-y (independently selected) = 1,  
 when a, c (independently selected) is 1;  
 R = PEG.

FIG. 51C



234/498

Fungi expressed Remicade.

e, g, i, r, t (independently selected) = 0 or 1;

a-d, f, h, j-m, n, s, u-y = 0; q, z = 1.

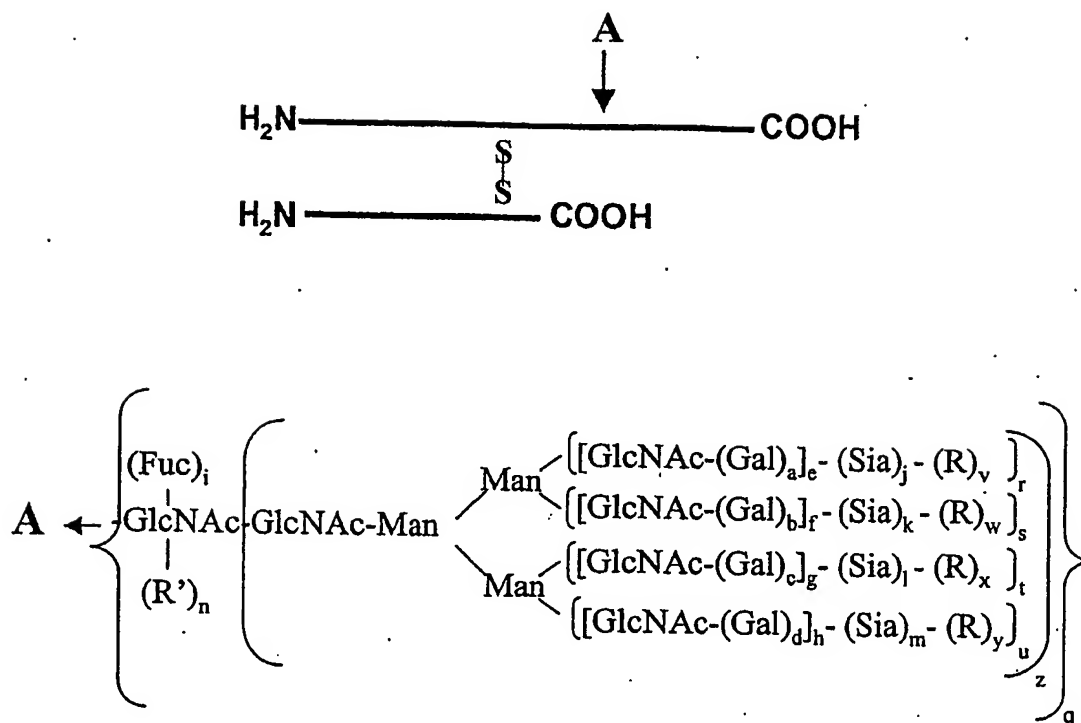
- ↓
1. Endo-H
  2. Galactosyltransferase, UDP-Gal
  - 3.. CMP-SA-radioisotope complex, ST3Gal3

a-m, r-z = 0; q, n = 1;

R' = -Gal-Sia-radioisotope complex.

FIG. 51D

235/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 or 1.

n, v-y = 0; z = 0 or 1;

R = modifying group, mannose, oligo-mannose;

R' = H, glycosyl residue, modifying group,  
glycoconjugate.

FIG. 52A

236/498

CHO, BHK, 293 cells, Vero expressed Reopro.  
 a-m, r-u (independently selected) = 0 or 1;  
 n = 0; v-y = 0; z = 1.

- ↓
1. Sialidase
  2. CMP-SA-PEG, ST3Gal3

a-m, r-u (independently selected) = 0 or 1;  
 v-y (independently selected) = 1,  
 when j-m (independently selected) is 1;  
 n = 0; R = PEG; z = 1.

FIG. 52B

Insect cell expressed Reopro.  
 a-h, j-n, s-y = 0; i, r (independently selected) = 0 or 1;  
 z = 1.

- ↓
1. GNT's 1&2, UDP-GlcNAc-PEG

a-d, f, h, j-n, s, u, w, y = 0;  
 e, g, i, r, t, v, x (independently selected) = 0 or 1;  
 v, x (independently selected) = 1,  
 when e, g (independently selected) is 1;  
 z = 1; R = PEG.

FIG. 52C

237/498

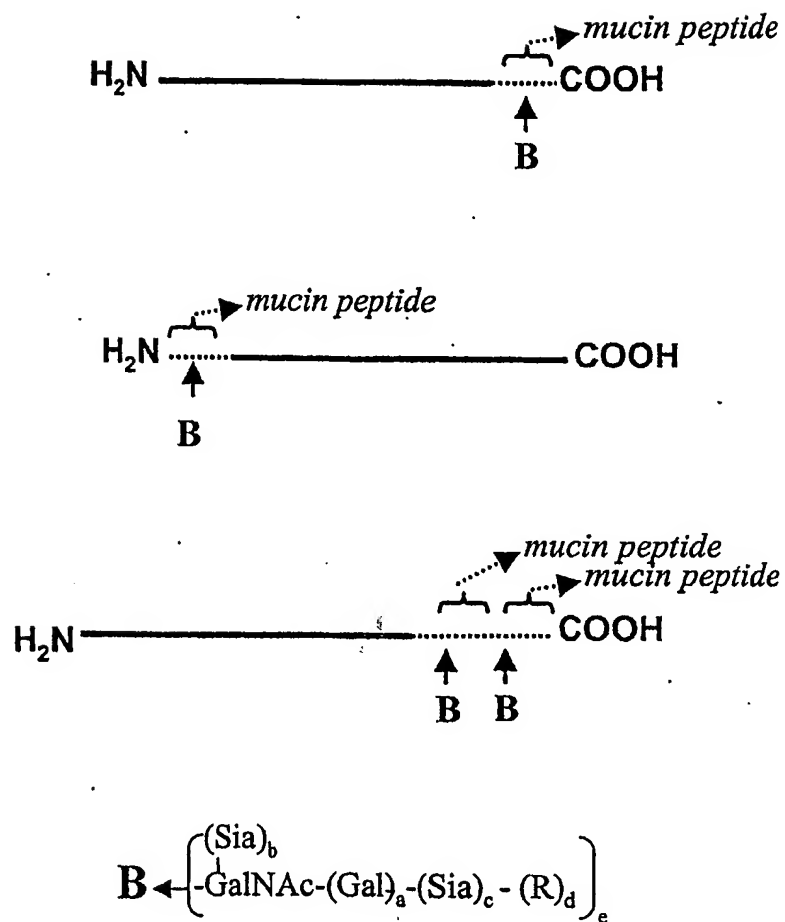
Yeast expressed Reopro.  
a-n = 0; r-y (independently selected) = 0 to 1;  
z = 1;  
R (branched or linear) = Man, oligomannose or  
polysaccharide.

1. Endo-H  
2. Galactosyltransferase, UDP-Gal-PEG

a-m, r-z = 0; n = 1; R' = -Gal-PEG.

FIG. 52D

238/498



a-c, e (independently selected) = 0 or 1;  
 d = 0; R = polymer

FIG. 52E

239/498

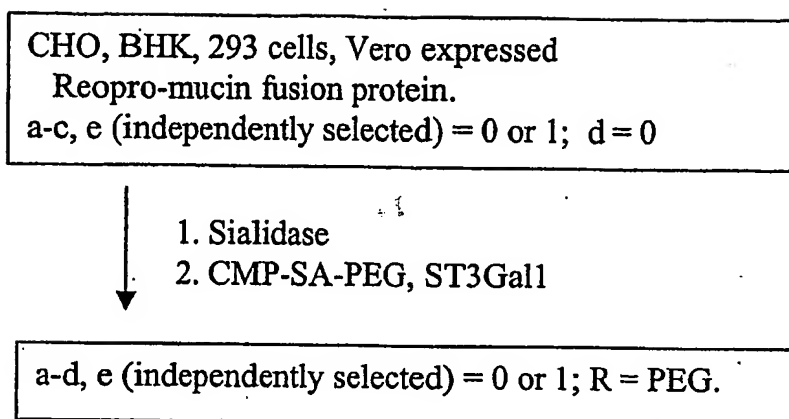


FIG. 52F

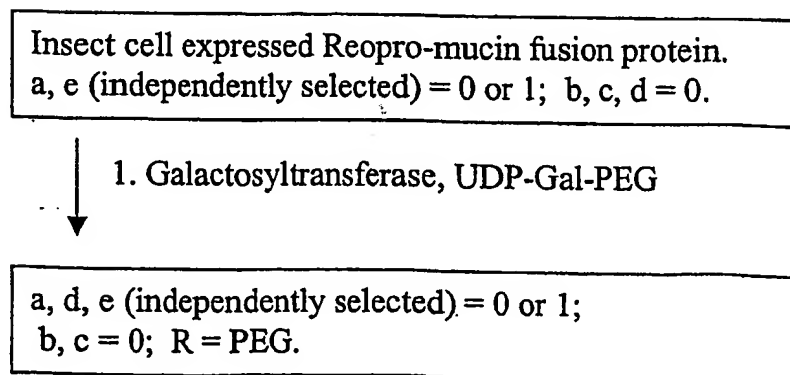


FIG. 52G

240/498

E. coli expressed Reopro-mucin fusion protein.  
a-e = 0.

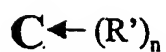
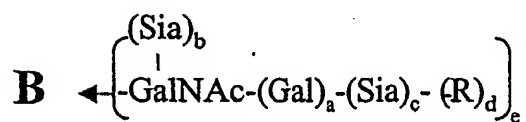
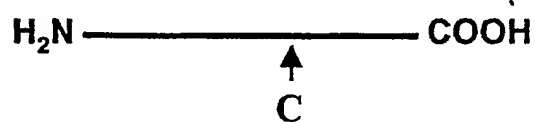
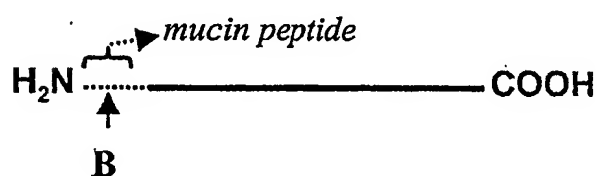
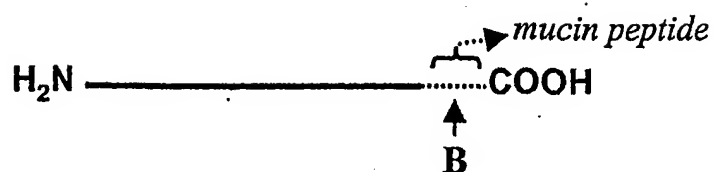


1. GalNAc Transferase, UDP-GalNAc
2. CMP-SA-PEG, sialyltransferase

c, d, e (independently selected) = 0 or 1;  
a, b = 0; R = PEG.

FIG. 52H

241/498



a-c, e (independently selected) = 0 or 1;  
d = 0; R = polymer, linker.

FIG. 52I



242/498

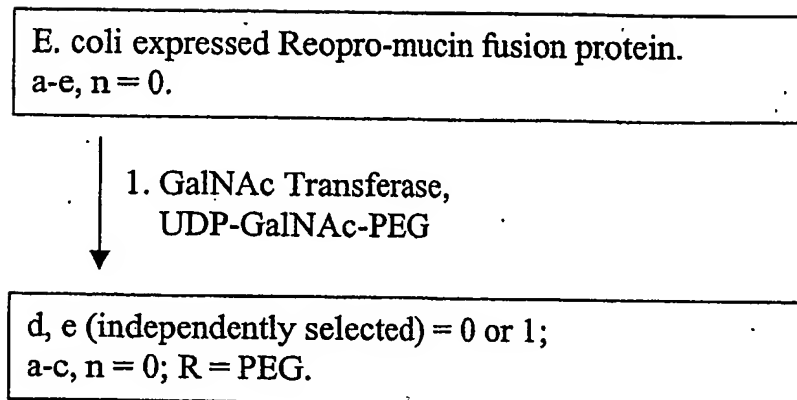


FIG. 52J

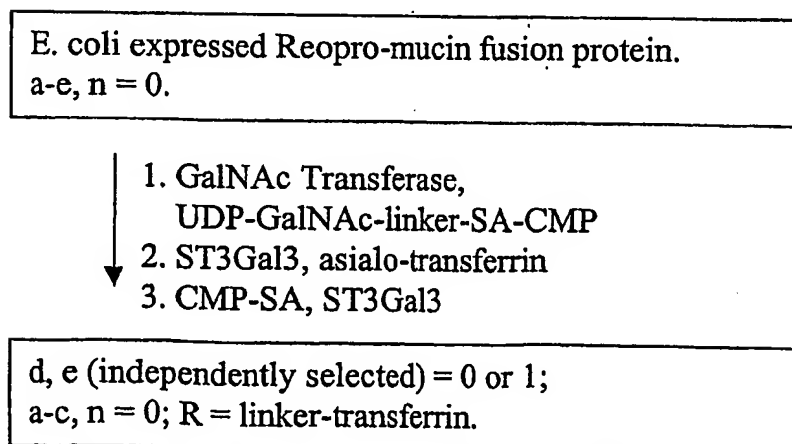


FIG. 52K

243/498

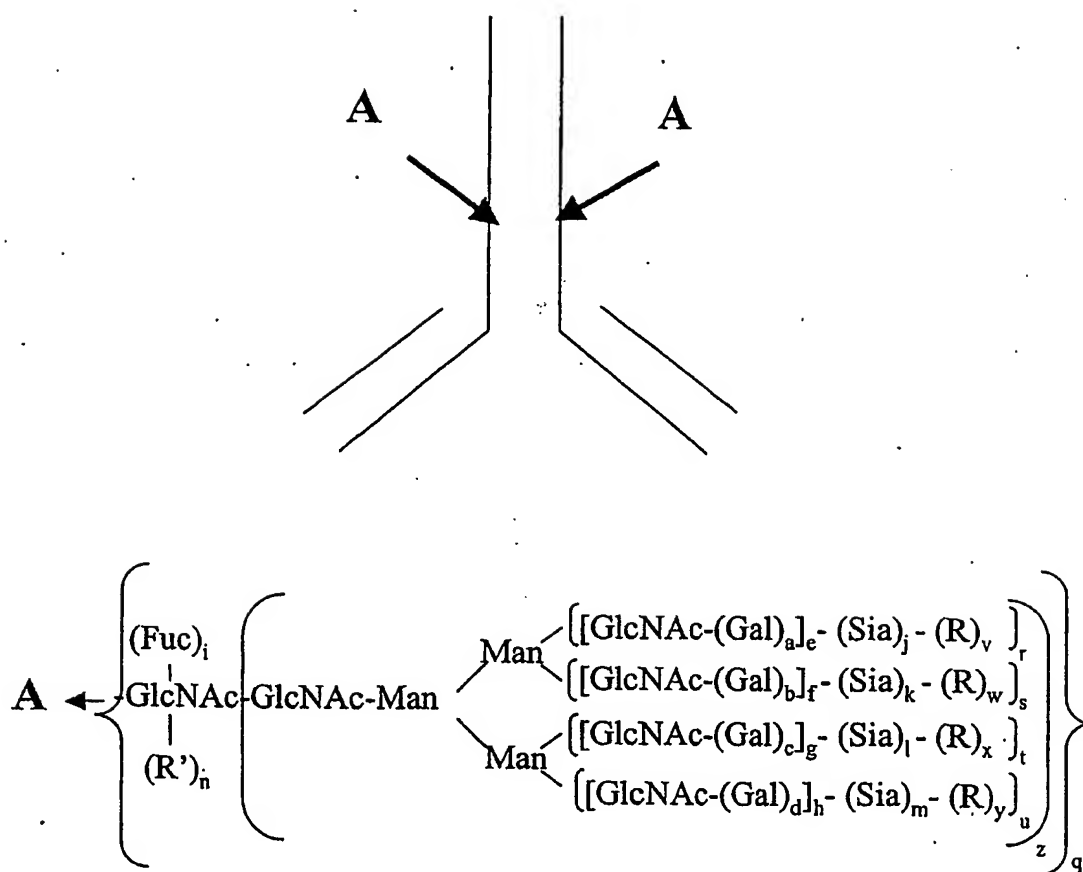
E. coli expressed Reopro(N)—no mucin peptide.  
a-e, n = 0.

- ↓
1. NHS-CO-linker-SA-CMP
  2. ST3Gal3, asialo-transferrin
  3. CMP-SA, ST3Gal3

a-e = 0; n = 1; R' = linker-transferrin.

FIG. 52L

244/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 or 1.

n, v-y = 0; z = 0 or 1; R = polymer, toxin, radioisotope-complex, drug, glycoconjugate.

R' = H, sugar, glycoconjugate.

z

FIG. 53A

245/498

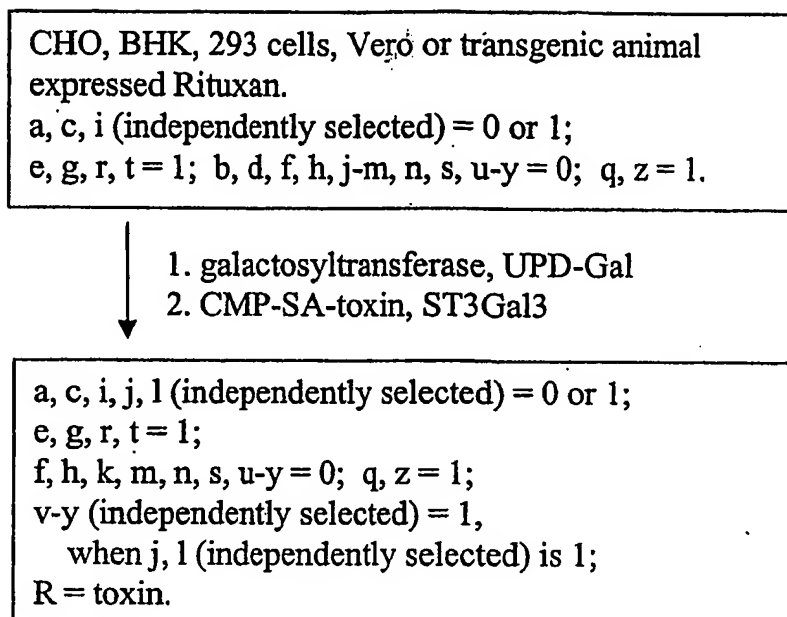


FIG. 53B

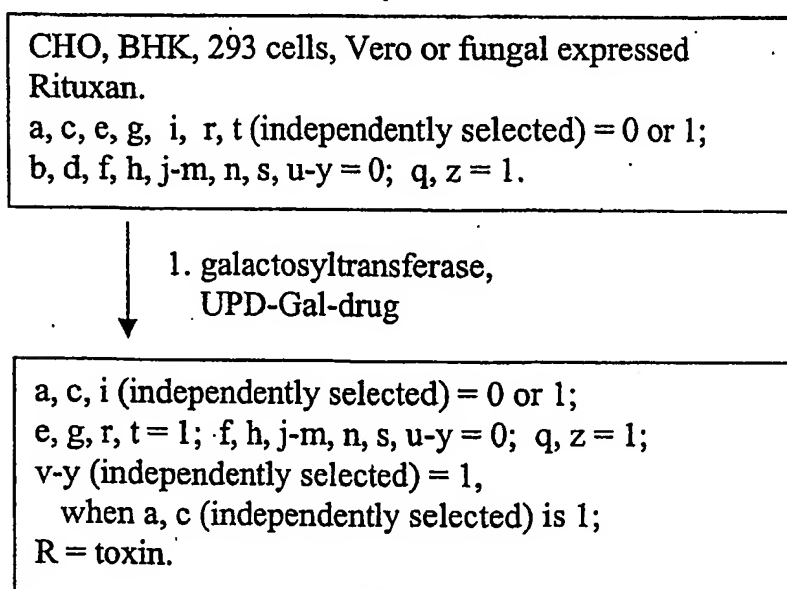


FIG. 53C

246/498

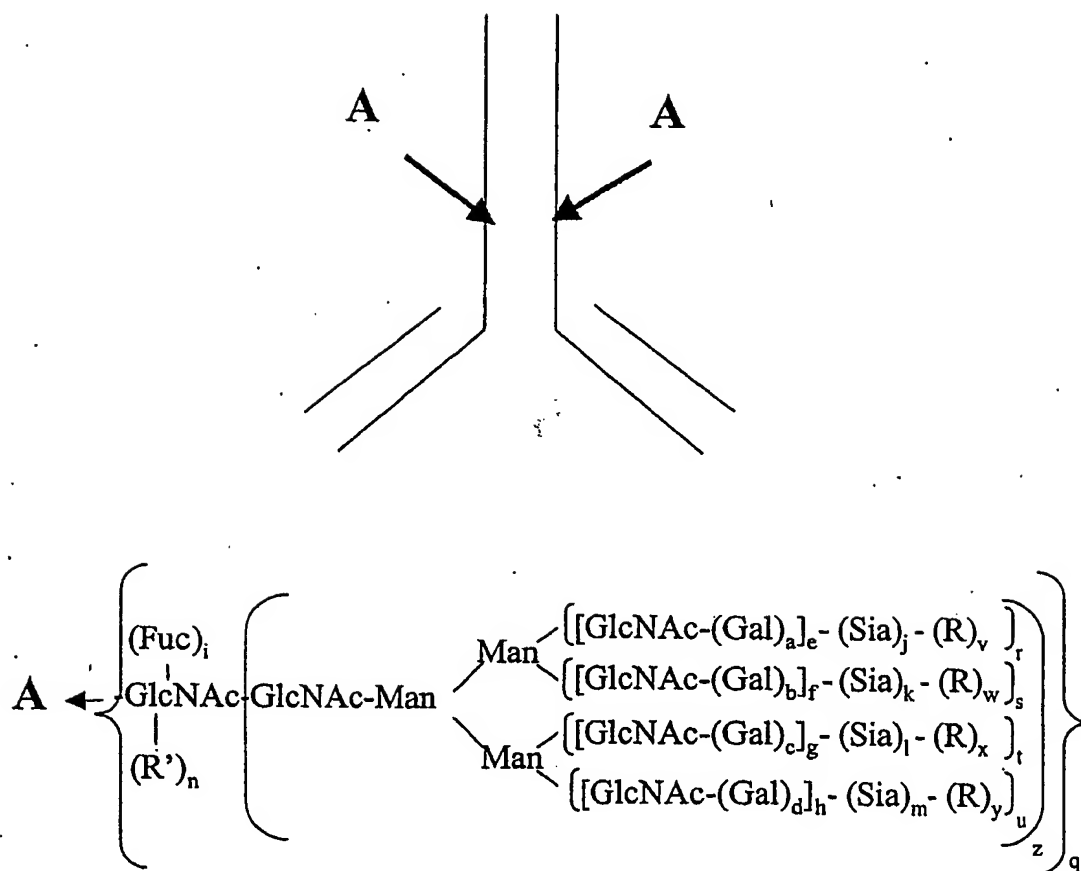
Fungi expressed Rituxan.  
e, g, i, r, t (independently selected) = 0 or 1;  
a-d, f, h, j-m, n, s, u-y = 0; q, z = 1.

- 1. Endo-H
- 2. Galactosyltransferase, UDP-Gal
- ↓ 3. CMP-SA-radioisotope complex, ST3Gal3

a-m, r-z = 0; q, n = 1;  
R' = -Gal-Sia-radioisotope complex.

FIG. 53D

247/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 or 1.

n, v-y = 0; z = 0 or 1;

R = polymer, toxin, radioisotope-complex, drug,  
glycoconjugate, mannose, oligo-mannose.

R' = H, glycosyl residue, modifying group, glycoconjugate.

FIG. 53E

248/498

CHO, BHK, 293 cells, Vero or transgenic animal  
expressed Rituxan.

a, c, i (independently selected) = 0 or 1;  
e, g, r, t = 1; b, d, f, h, j-m, n, s, u-y = 0;  
q, z = 1.



1. galactosyltransferase, UDP-Gal
2. CMP-SA-PEG, ST3Gal3

a, c, i, j, l (independently selected) = 0 or 1;  
e, g, r, t = 1; f, h, k, m, n, s, u-y = 0;  
q, z = 1; v-y (independently selected) = 1,  
when j, l (independently selected) is 1;  
R = PEG.

FIG. 53F

Fungi, yeast or CHO expressed Rituxan.

e, g, i, r, t, v, x (independently selected) = 0 or 1;  
a-d, f, h, j-m, n, s, u, w, y = 0; q, z = 1;  
R (independently selected) = mannose, oligomannose,  
polymannose.

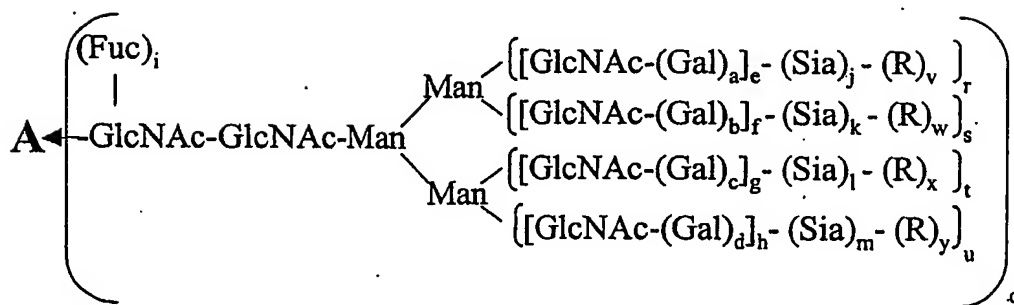
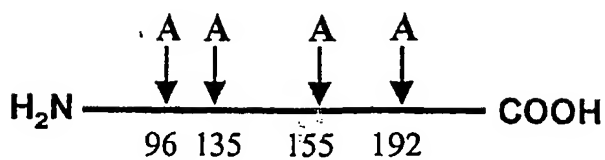


1. mannosidases (alpha and beta)
2. GNT-I,II, UDP-GlcNAc
3. Galactosyltransferase, UDP-Gal-radioisotope

a-m, r-z = 0; q, n = 1;  
R' = -Gal-radioisotope complex.

FIG. 53G

249/498



a-d, i, q-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0; R = mannose, polymer.

FIG. 54A



250/498

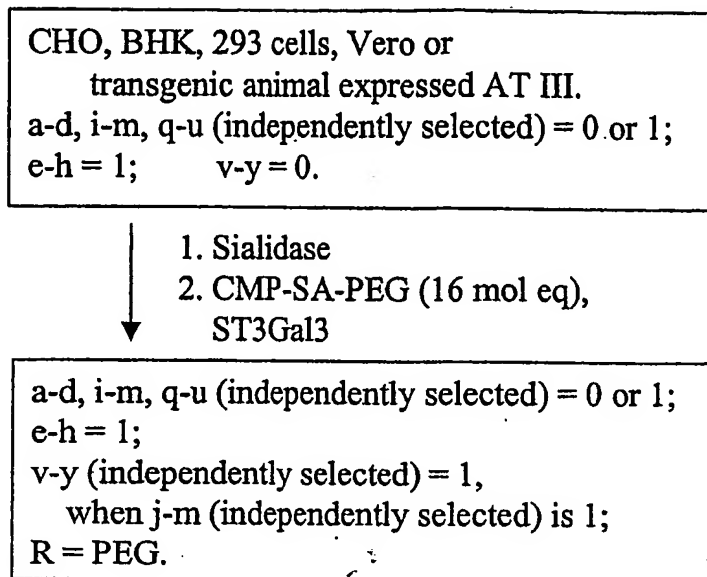


FIG. 54B

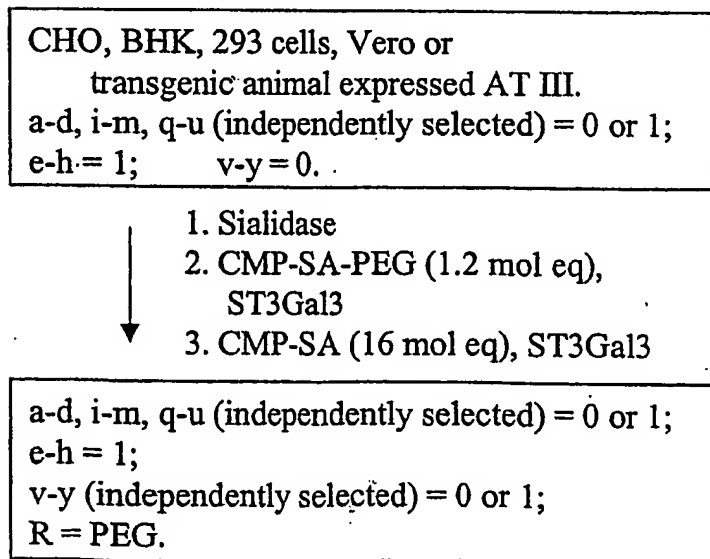


FIG. 54C

251/498

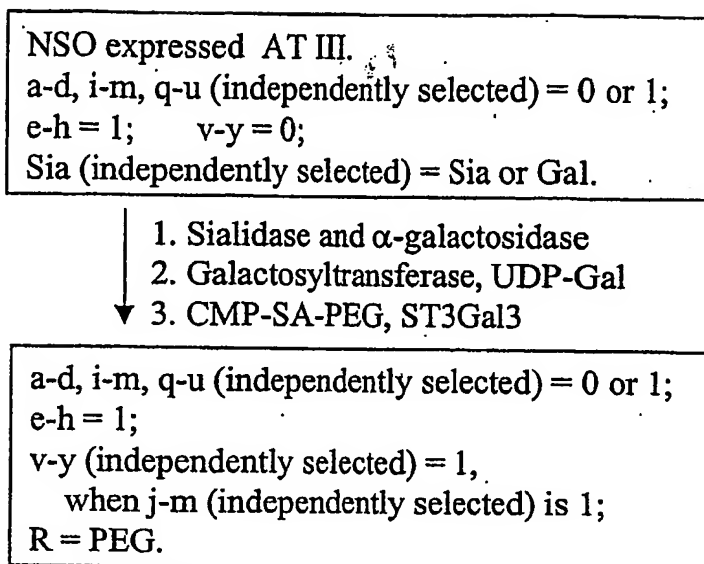


FIG. 54D

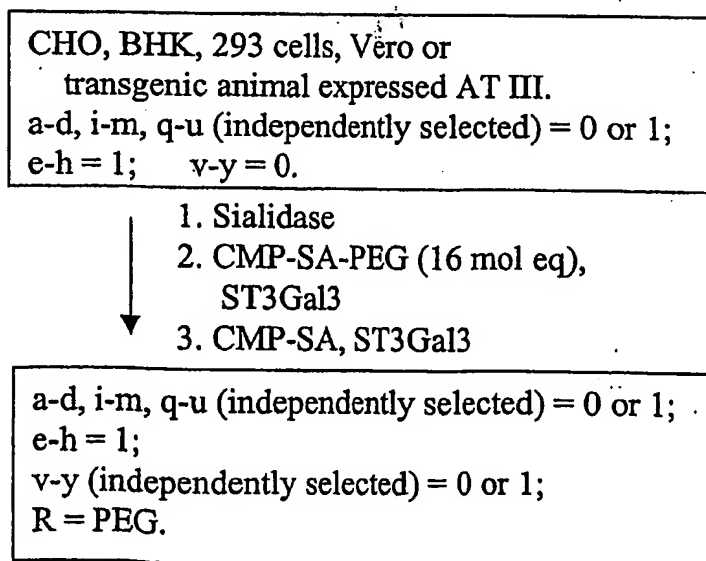


FIG. 54E

252/498

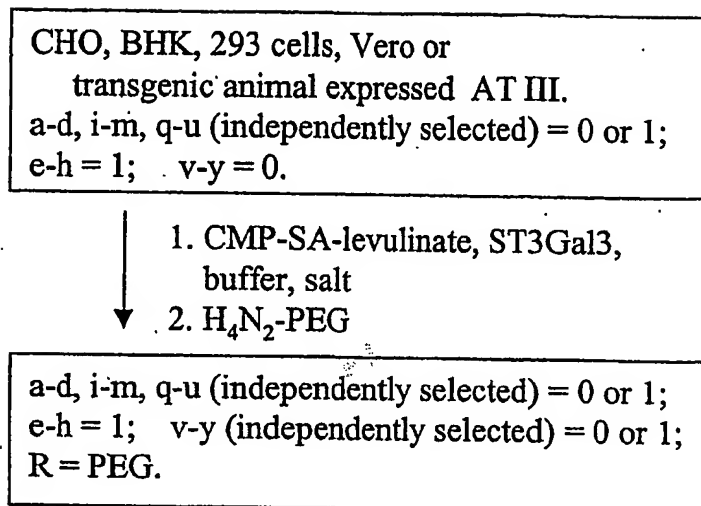


FIG. 54F

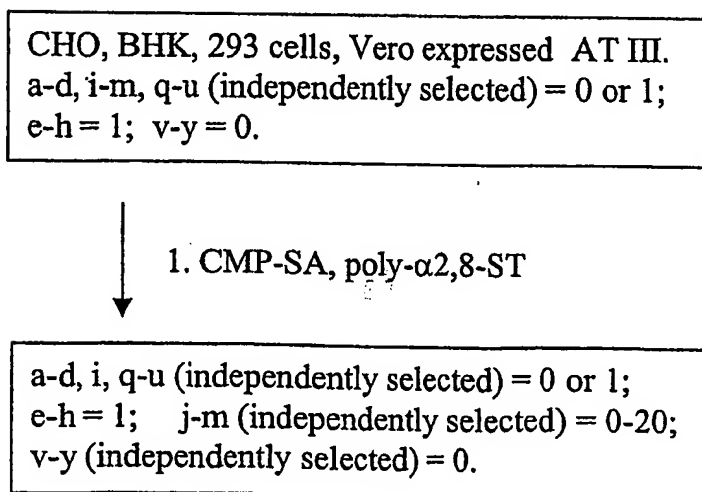
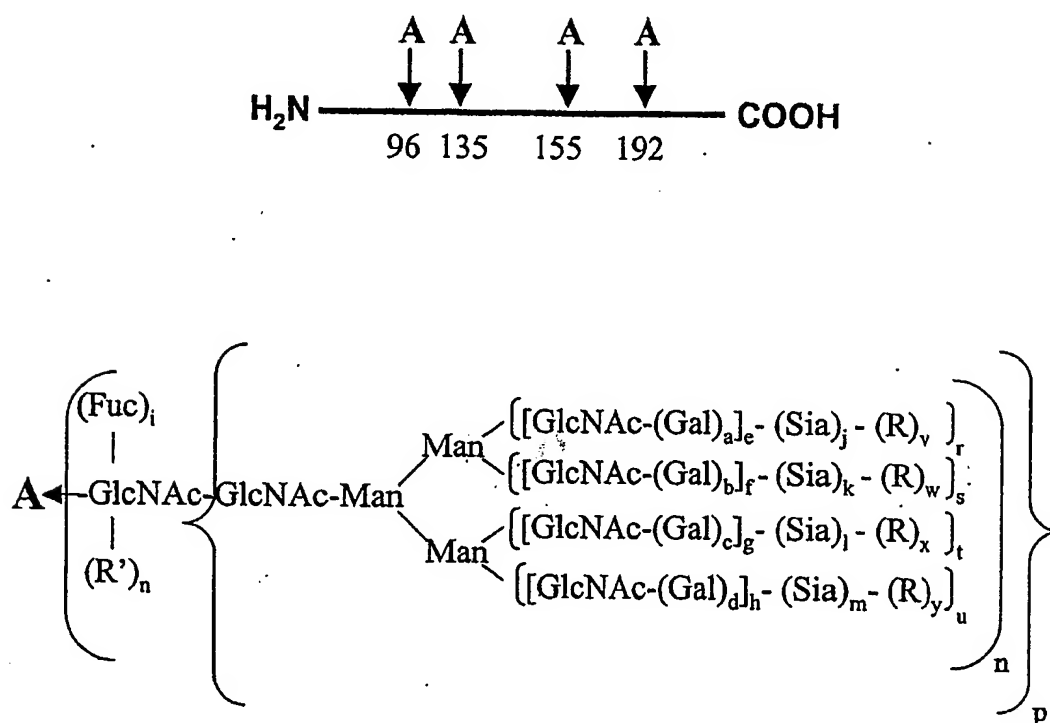


FIG. 54G

253/498



a-d, i, p-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

$$v-y = 0 \text{ to } 100.$$

R = polymer, linker, mannose.

R' = H, sugar, glycoconjugate.

FIG. 54H

254/498

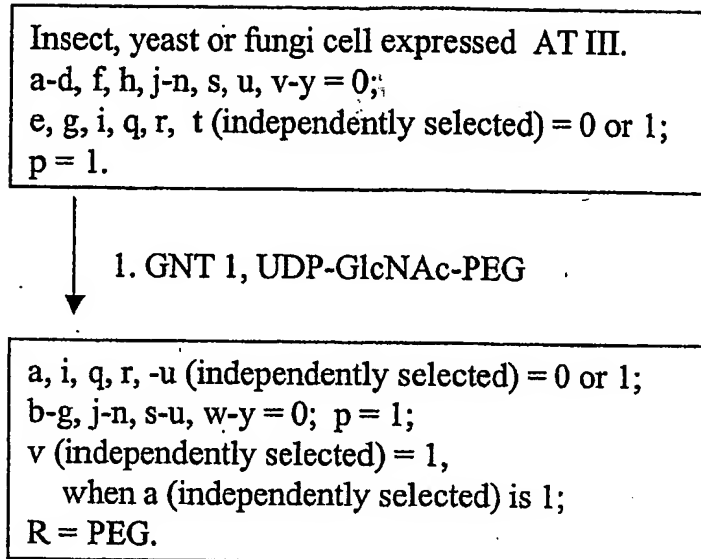


FIG. 54I

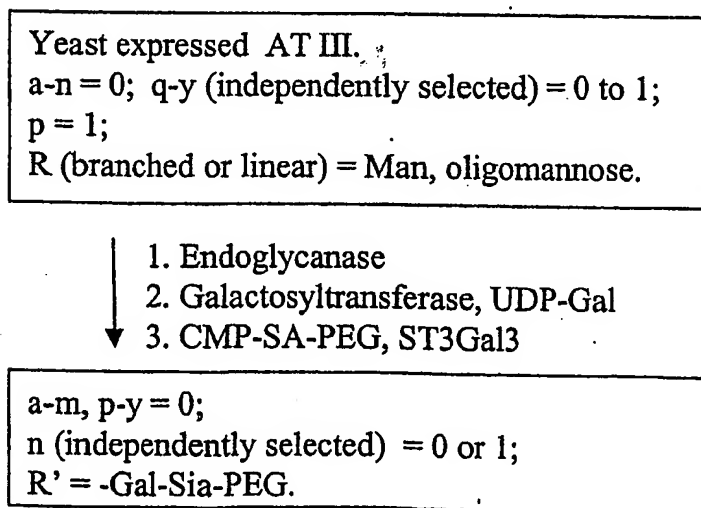


FIG. 54J

255/498

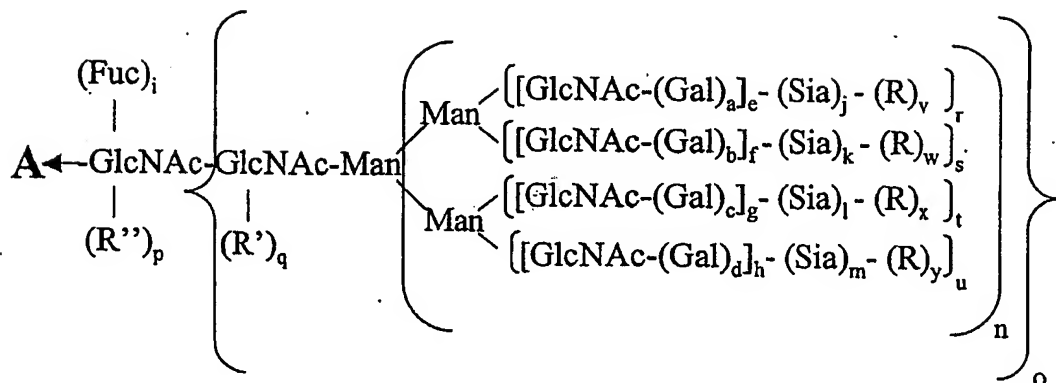
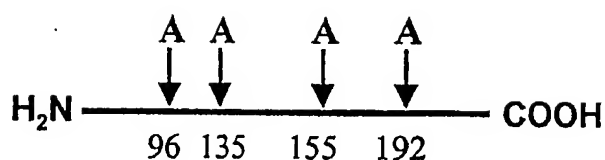
CHO, BHK, 293 cells, Vero expressed AT III.  
a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y = 0.

- ↓
1. CMP-SA-linker-Gal-UDP,  
ST3Gal3
  2. Galactosyltransferase, transferrin  
treated with endoglycanase

a-m, q-u (independently selected) = 0 or 1;  
p = 1; n = 0;  
v-y (independently selected) = 0 or 1;  
R = linker-transferrin.

FIG. 54K

256/498



a-d, i, n-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 to 20.

R = polymer.

R', R'' (independently selected) = sugar, glycoconjugate.

FIG. 54L

257/498

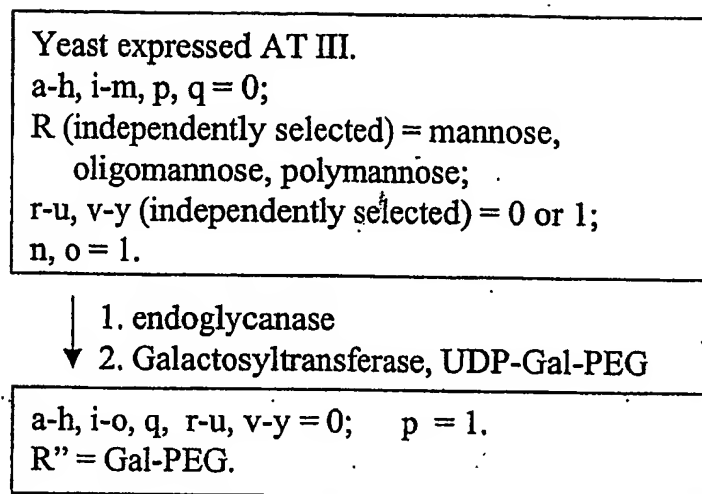


FIG. 54M

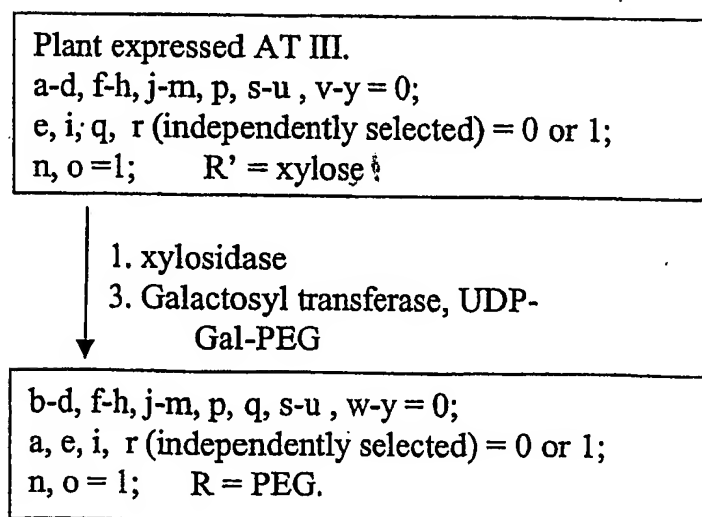


FIG. 54N



258/498

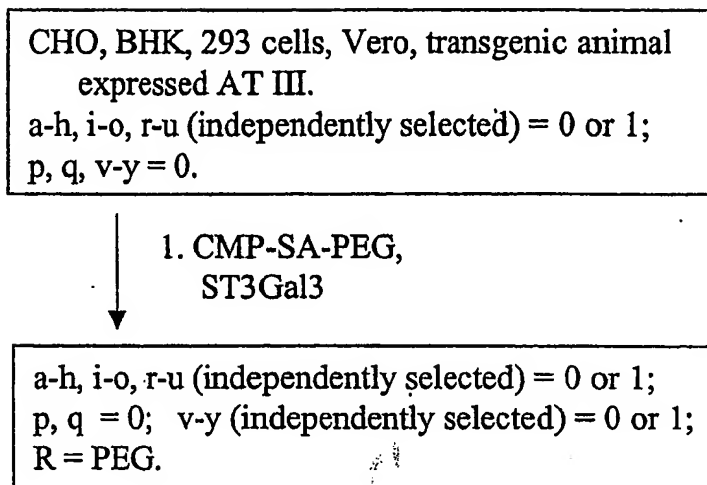
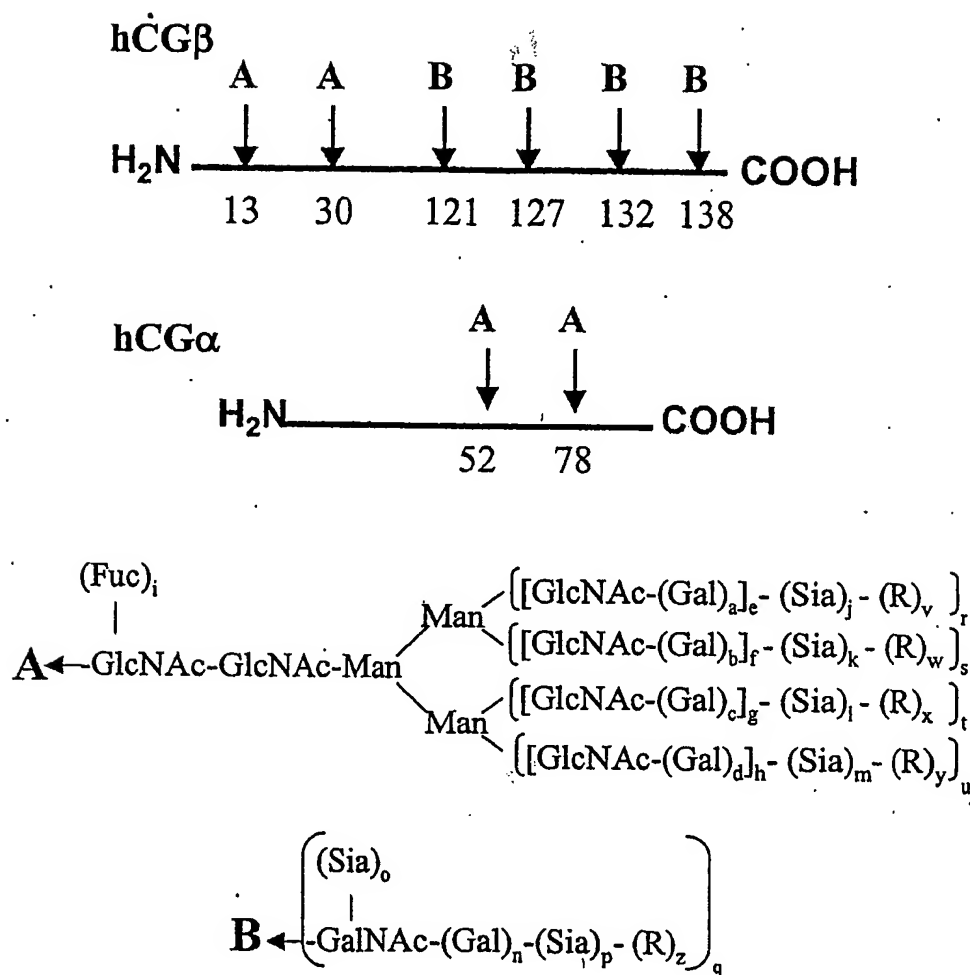


FIG. 540

259/498



a-d, i, n-u (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 4.

j-m (independently selected) = 0 to 20.

v-z = 0; R = polymer

FIG. 55A

260/498

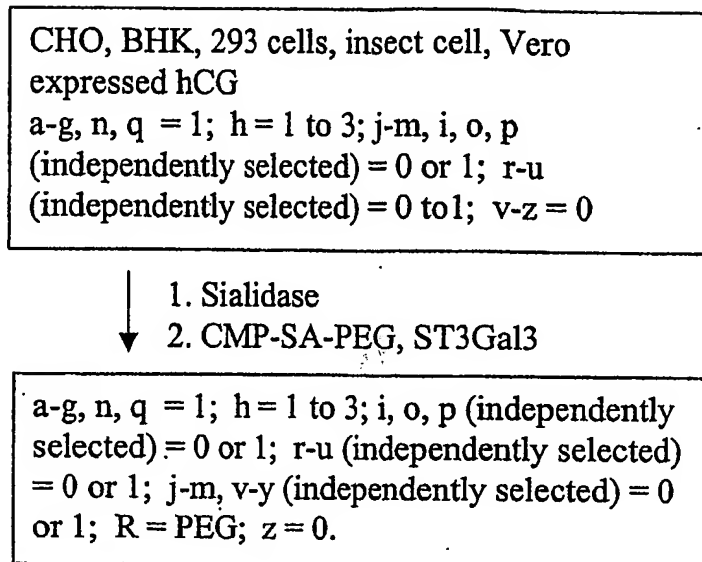


FIG. 55B

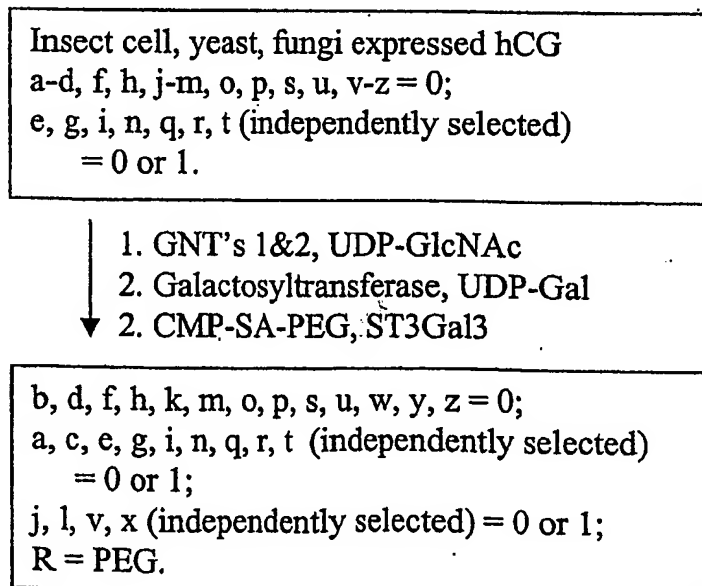


FIG. 55C

261/498

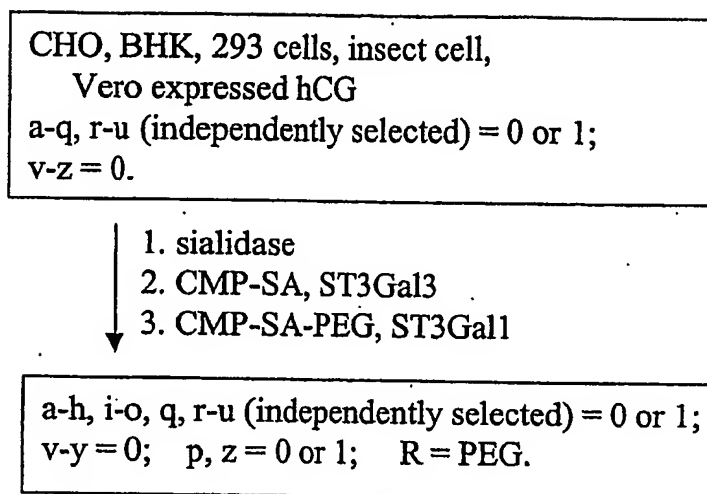


FIG. 55D

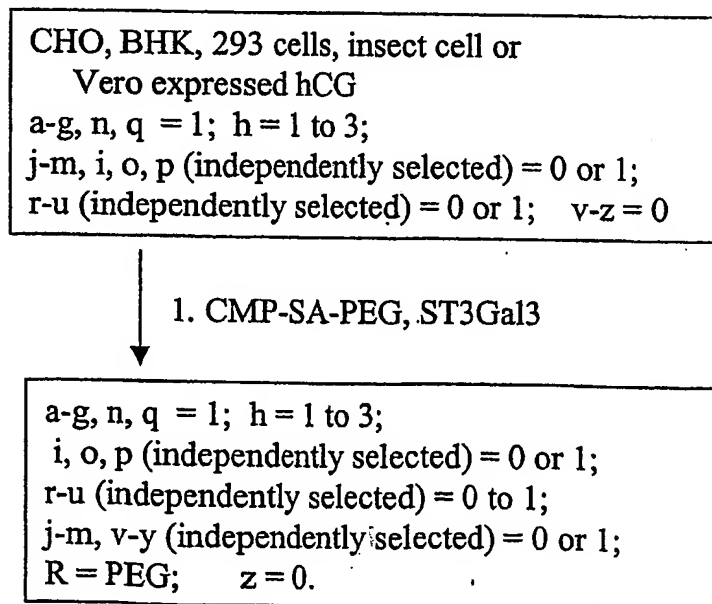


FIG. 55E

262/498

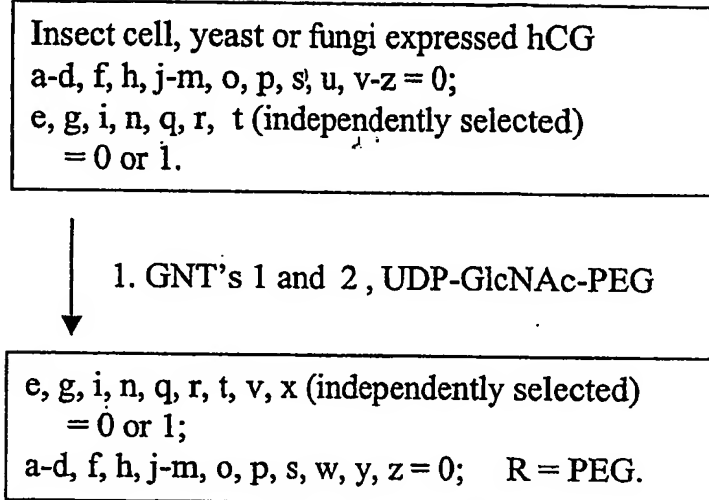


FIG. 55F

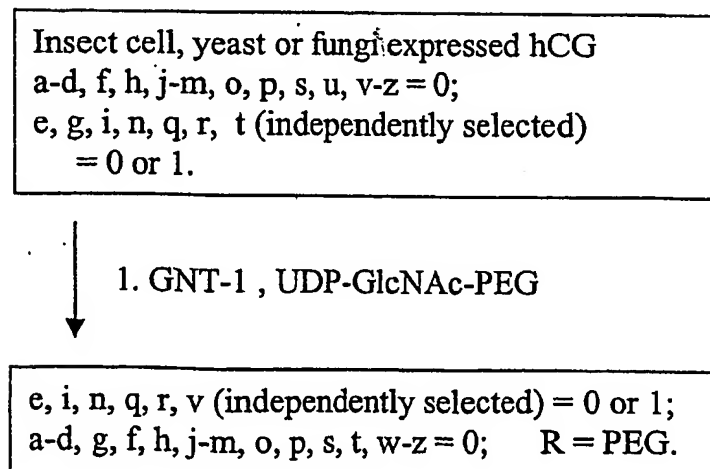


FIG. 55G

263/498

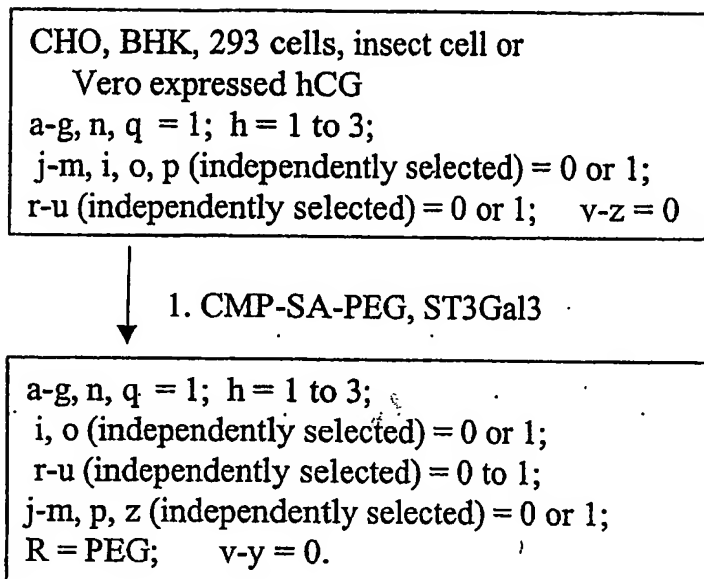


FIG. 55H

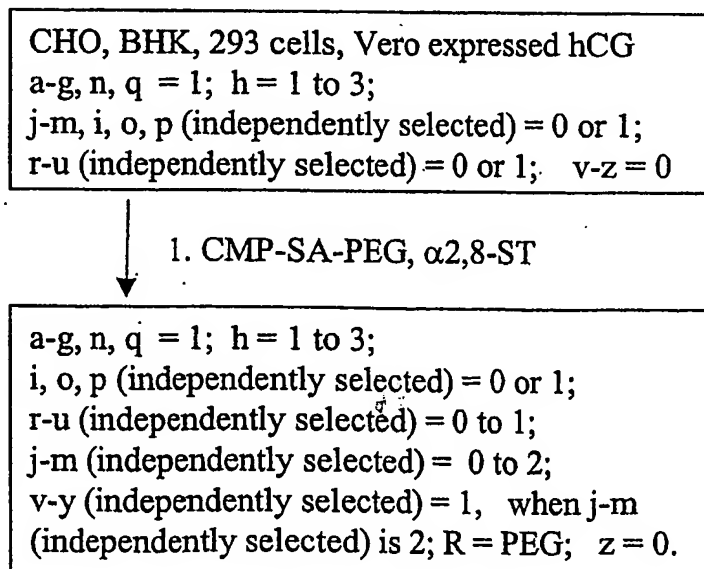


FIG. 55I

264/498

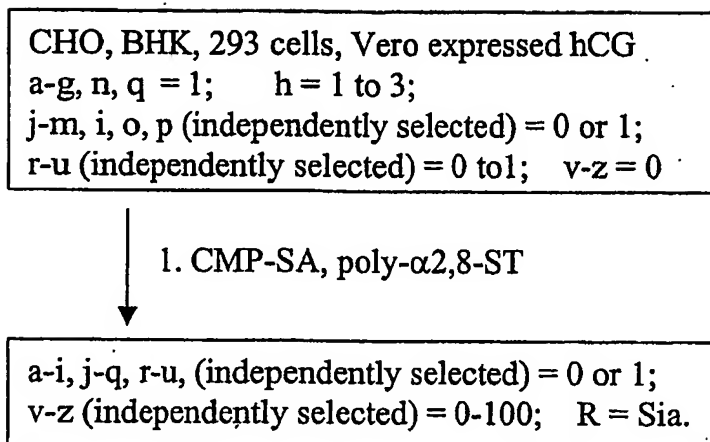
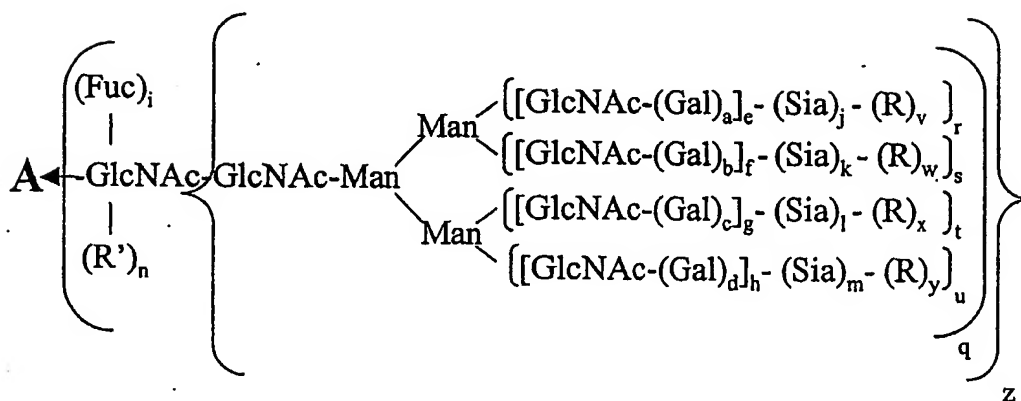
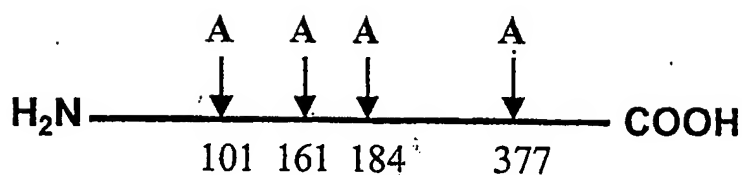


FIG. 55J

265/498



a-d, i, n, q-u, z (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0 to 100;

R = mannose, mannose-6-phosphate and mannose, polymer.

FIG. 56A



266/498

CHO, BHK, 293 cells, insect cells, Vero expressed  
and secreted alpha-galactosidase  
a-h, i-m, q-u (independently selected) = 0 or 1;  
z = 1; n, v-y = 0; and when a-n = 0, then r-u (independently  
selected) = 0 or 1; v-y (independently selected) = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

- ↓  
1. Endo-H  
2. Galactosyltransferase, UDP-Gal-PEG-transferrin

a-h, i-m, q-u (independently selected) = 0 or 1;  
n, v-y = 0; z = 1; and when z = 0 and q = 1,  
then n (independently selected) = 0 or 1;  
R' = Gal-PEG-transferrin.

FIG. 56B

CHO, BHK, 293 cells, Insect cells,  
Vero expressed and secreted alpha-galactosidase  
a-h, i-m, q-u (independently selected) = 0 or 1; z = 1; n, v-y  
= 0; and when a-n = 0, then r-u (independently selected) = 0  
or 1; v-y (independently selected) = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

- ↓  
1. Sialidase  
2. CMP-SA-linker-Mannose-6-phosphate  
ST3Gal3

a-h, i-m, q-u, v-y (independently selected) = 0 or 1;  
n = 0; z = 1; R = mannose-6-phosphate; and when a-n  
= 0, then r-u (independently selected) = 0 or 1;  
v-y (independently selected) = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

FIG. 56C

267/498

NSO expressed alpha-galactosidase.

a-d, i-m, q-u (independently selected) = 0 or 1;

e-h = 1; v-y = 0;

Sia (independently selected) = Sia or Gal.

- ↓
1. Sialidase and  $\alpha$ -galactosidase
  2. Galactosyltransferase, UDP-Gal
  3. CMP-SA-linker-mannose-6-phosphate  
sialyltransferase

a-d, i-m, q-u (independently selected) = 0 or 1; e-h = 1;  
v-y (independently selected) = 1, when j-m (independently  
selected) is 1; R = mannose-6 phosphate

FIG. 56D

CHO, BHK, 293 cells, Insect cells, Vero expressed and  
secreted alpha-galactosidase

a-h, i-m, q-u (independently selected) = 0 or 1; z = 1;

n, v-y = 0; and when a-n = 0, then r-u (independently  
selected) = 0 or 1; v-y (independently selected) = 0-100;

R = mannose or mannose with mannose-6-phosphate.

- ↓
1. Sialidase
  2. CMP-SA-PEG, sialyltransferase

a-h, i-m, q-u, v-y (independently selected) = 0 or 1; n = 0; z  
= 1; R = PEG; and when a-n = 0, then r-u (independently  
selected) = 0 or 1; v-y = 0-100; R = mannose or mannose  
with mannose-6-phosphate.

FIG. 56E

268/498

CHO, BHK, 293 cells, Insect cells, Vero, yeast, fungi  
expressed alpha-galactosidase.

a-i, v-y = 0; q (independently selected) = 0 or 1; z = 1;

r-u (independently selected) = 0 or 1;

j-m (independently selected) = 0-100;

Sia = mannose or mannose with mannose-6-phosphate.

↓ 1. mannosyltransferase,  
GDP-mannose-linker-ApoE

a-i = 0; q (independently selected) = 0 or 1; z = 1;

r-u (independently selected) = 0 or 1;

j-m (independently selected) = 0-100;

Sia = mannose or mannose with mannose-6-phosphate;

v-y (independently selected) = 0 or 1;

R = mannose-linker-ApoE.

FIG. 56F

CHO, BHK, 293 cells, Insect cells, Vero, yeast,  
fungi expressed alpha-galactosidase.

a-i, v-y = 0; q (independently selected) = 0 or 1; z = 1;

r-u (independently selected) = 0 or 1;

j-m (independently selected) = 0-100;

Sia = mannose or mannose with mannose-6-phosphate.

↓ 1. endo-H  
2. galactosyltransferase,  
UDP-Gal-linker-alpha2-macroglobulin

a-m, r-z = 0; n, q (independently selected) = 0 or 1;

R' = galactose-linker-alpha2-macroglobulin.

FIG. 56G

269/498

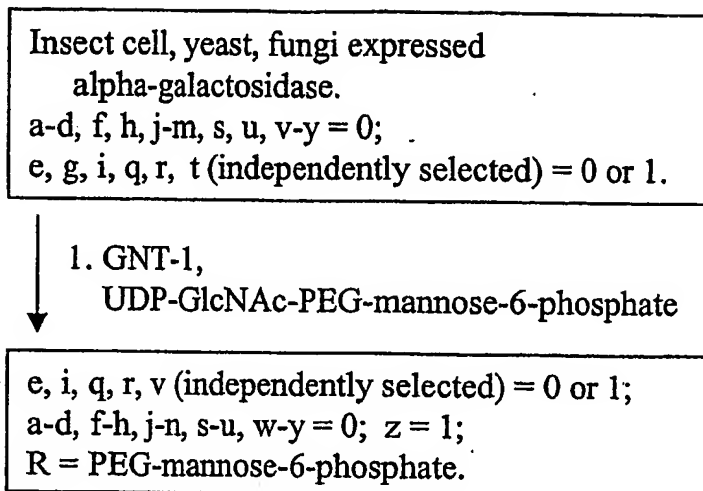


FIG. 56H

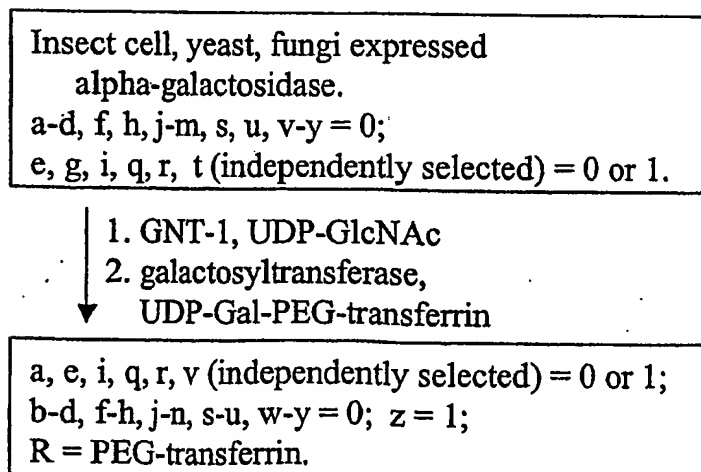


FIG. 56I

270/498

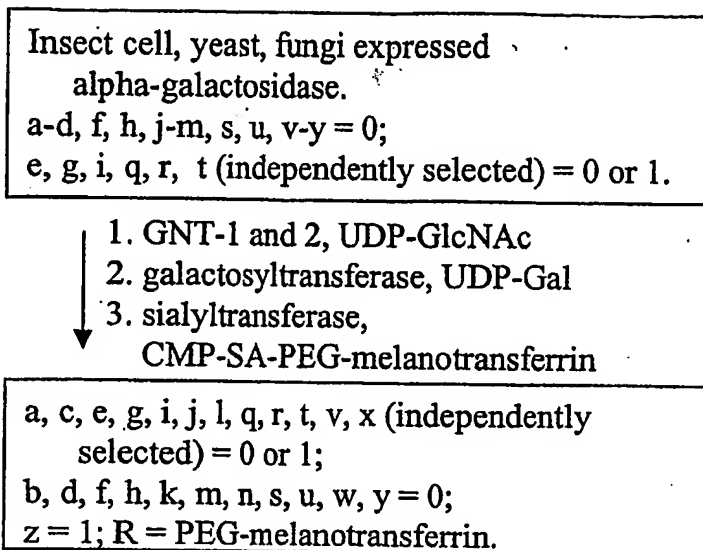
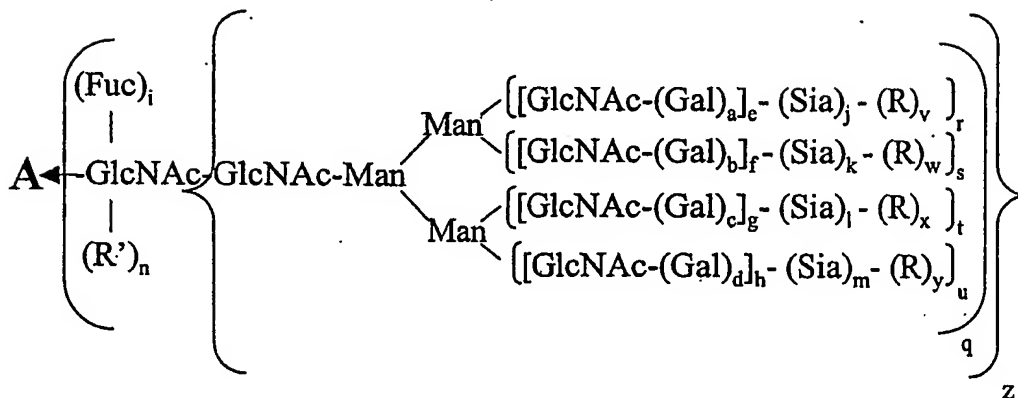
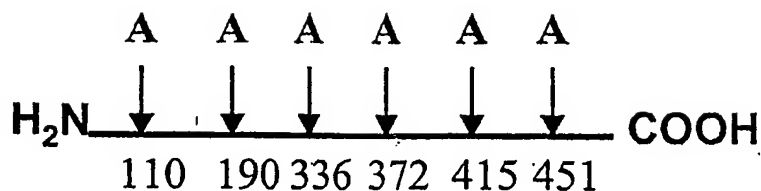


FIG. 56J

271/498



a-d, i, n, q-u, z (independently selected) = 0 or 1.

e-h (independently selected) = 0 to 6.

j-m (independently selected) = 0 to 100.

v-y = 0 to 100;

R = mannose, mannose-6-phosphate and mannose, polymer.

FIG. 57A

272/498

CHO, BHK, 293 cells, Insect cells, Vero expressed  
and secreted alpha-iduronidase  
a-h, i-m, q-u (independently selected) = 0 or 1; z = 1;  
n, v-y = 0; and when a-n = 0, then r-u (independently  
selected) = 0 or 1; v-y (independently selected) = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

- ↓  
1. Endo-H  
↓  
2. Galactosyltransferase, UDP-Gal-PEG-transferrin

a-h, i-m, q-u (independently selected) = 0 or 1;  
n, v-y = 0; z = 1; and when z = 0 and q = 1, then n  
(independently selected) = 0 or 1; R' = Gal-PEG-transferrin.

FIG. 57B

CHO, BHK, 293 cells, Insect cells, Vero expressed  
and secreted alpha-iduronidase  
a-h, i-m, q-u (independently selected) = 0 or 1; z = 1;  
n, v-y = 0; and when a-n = 0, then r-u (independently  
selected) = 0 or 1; v-y (independently selected) = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

- ↓  
1. Sialidase  
↓  
2. CMP-SA-linker-Mannose-6-phosphate ST3Gal3

a-h, i-m, q-u, v-y (independently selected) = 0 or 1; n = 0;  
z = 1; R = mannose-6-phosphate; and when a-n = 0,  
then r-u (independently selected) = 0 or 1;  
v-y (independently selected) = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

FIG. 57C

273/498

NSO expressed alpha-iduronidase.

a-d, i-m, q-u (independently selected) = 0 or 1; e-h = 1;  
v-y = 0; Sia (independently selected) = Sia or Gal.

- ↓
1. Sialidase and  $\alpha$ -galactosidase
  2. Galactosyltransferase, UDP-Gal
  - ↓ 3. CMP-SA-linker-mannose-6-phosphate sialyltransferase

a-d, i-m, q-u (independently selected) = 0 or 1;  
e-h = 1; v-y (independently selected) = 1,  
when j-m (independently selected) is 1;  
R = mannose-6 phosphate

FIG. 57D

CHO, BHK, 293 cells, Insect cells, Vero expressed  
and secreted alpha-iduronidase

a-h, i-m, q-u (independently selected) = 0 or 1; z = 1;  
n, v-y = 0; and when a-n = 0, then r-u (independently  
selected) = 0 or 1; v-y (independently selected) = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

- ↓
1. Sialidase
  2. CMP-SA-PEG, sialyltransferase

a-h, i-m, q-u, v-y (independently selected) = 0 or 1; n = 0;  
z = 1; R = PEG; and when a-n = 0, then r-u  
(independently selected) = 0 or 1; v-y = 0-100;  
R = mannose or mannose with mannose-6-phosphate.

FIG. 57E



274/498

CHO, BHK, 293 cells, Insect cells, Vero, yeast, fungi  
expressed alpha-iduronidase.

a-i, v-y = 0; q (independently selected) = 0 or 1; z = 1;

r-u (independently selected) = 0 or 1;

j-m (independently selected) = 0-100;

Sia = mannose or mannose with mannose-6-phosphate.

↓ 1. mannosyltransferase,  
GDP-mannose-linker-ApoE

a-i = 0; q (independently selected) = 0 or 1; z = 1;

r-u (independently selected) = 0 or 1; j-m (independently  
selected) = 0-100;

Sia = mannose or mannose with mannose-6-phosphate;

v-y (independently selected) = 0 or 1;

R = mannose-linker-ApoE.

FIG. 57F

CHO, BHK, 293 cells, Insect cells, Vero, yeast, fungi  
expressed alpha-iduronidase.

a-i, v-y = 0; q (independently selected) = 0 or 1;

z = 1; r-u (independently selected) = 0 or 1;

j-m (independently selected) = 0-100;

Sia = mannose or mannose with mannose-6-phosphate.

↓ 1. endo-H  
2. galactosyltransferase,  
UDP-Gal-linker-alpha2-macroglobulin

a-m, r-z = 0; n, q (independently selected) = 0 or 1;

R' = galactose-linker-alpha2-macroglobulin.

FIG. 57G

275/498

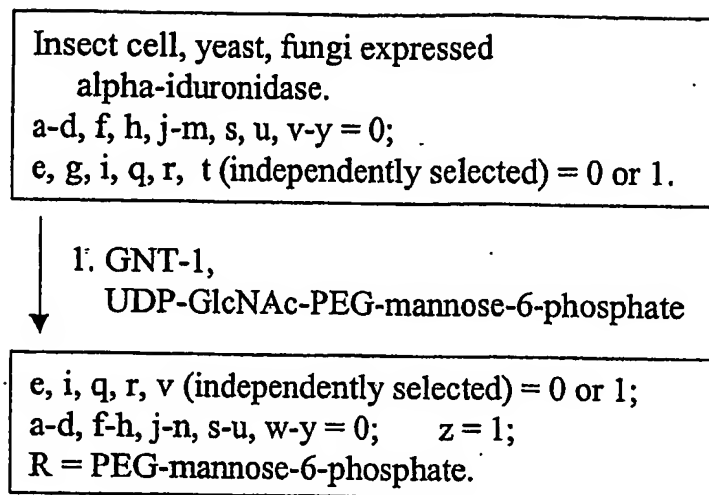


FIG. 57H

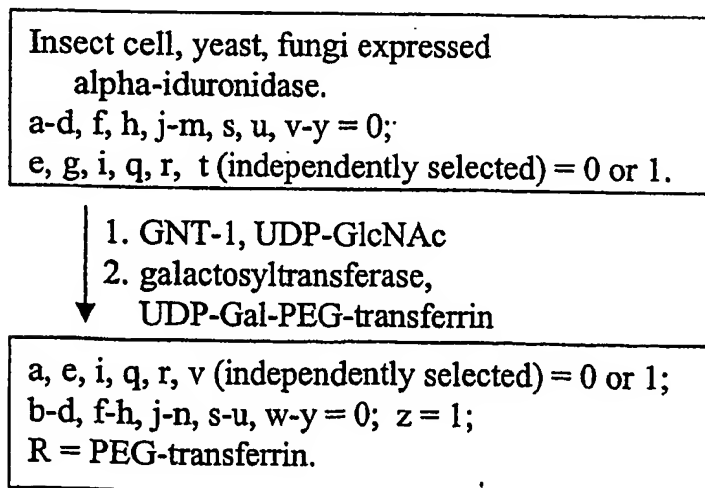


FIG. 57I

276/498

Insect cell, yeast, fungi expressed  
alpha-iduronidase.

a-d, f, h, j-m, s, u, v-y = 0;

e, g, i, q, r, t (independently selected) = 0 or 1.

- ↓  
1. GNT-1 and 2, UDP-GlcNAc  
2. galactosyltransferase, UDP-Gal  
3. sialyltransferase,  
CMP-SA-PEG-melanotransferrin

a, c, e, g, i, j, l, q, r, t, v, x

(independently selected) = 0 or 1;

b, d, f, h, k, m, n, s, u, w, y = 0; z = 1;

R = PEG-melanotransferrin.

FIG. 57J

277/498

FIG. 58A

ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAT  
GCTTAGAGCAAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAG  
GAGAAGCTGTGTGCCACCTACAAGCTGTGCCACCCCGAGGAGCTGGT  
GCTGCTCGGACACTCTCTGGGCATCCCCTGGGCTCCCCTGAGCAGCTG  
CCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCCAACTCCATA  
GCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCT  
CCCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCG  
ACTTTGCCACCACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCC  
CCTGCCCTGCAGCCCACCCAGGGTGCCATGCCGGCCTTCGCCTCTGCT  
TTCCAGCGCCGGGCAGGAGGGGTCTGTTGCCTCCCATCTGCAGAG  
CTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCCAGCCCTG  
A

FIG. 58B

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu  
Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr  
Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro  
Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser  
Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile  
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe  
Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro  
Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val  
Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His  
Leu Ala Gln Pro

278/498

FIG. 59A

GCGCCTCTTATGTACCCACAAAAATCTATTTTCAAAAAAGTTGCTCTA  
AGAATATAGTTATCAAGTTAAGTAAAATGTCAATAGCCTTTTAATTTA  
ATTTTAAATTGTTTTATCATTCTTTGCAATAATAAACATTAACTTTAT  
ACTTTTAAATTTAATGTATAGAATAGAGATATACATAGGATATGTAAA  
TAGATACACAGTGTATATGTGATTAAAATATAATGGGAGATTCAATC  
AGAAAAAAGTTTCTAAAAAGGCTCTGGGGTAAAAGAGGAAGGAAAC  
AATAATGAAAAAAATGTGGTGAGAAAAACAGCTGAAAACCCATGTA  
AAGAGTGTATAAAGAAAGCAAAAAGAGAAGTAGAAAGTAACACAGG  
GGCATTGGAAGTGTAAACGAGTATGTTCCCTATTTAAGGCTAGGC  
ACAAAGCAAGGTCTTCAGAGAACCTGGAGCCTAAGGTTTAGGCTCAC  
CCATTTCAACCAGTCTAGCAGCATCTGCAACATCTACAATGGCCTTGA  
CCTTTGCTTTACTGGTGGCCCTCCTGGTGCTCAGCTGCAAGTCAAGCT  
GCTCTGTGGGCTGTGATCTGCCTCAAACCCACAGCCTGGGTTAGCAGG  
AGGACCTTGATGCTCCTGGCACAGATGAGGAGAATCTCTCTTTTCTCC  
TGCTTGAAGGACAGACATGACTTTGGATTTCCCCAGGAGGAGTTTGG  
CAACCAGTTCCAAAAGGCTGAAACCATCCCTGTCCTCCATGAGATGA  
TCCAGCAGATCTTCAATCTCTTCAGCACAAAGGACTCATCTGCTGCTT  
GGGATGAGACCCTCCTAGACAAATTCTACACTGAACTCTACCAGCAG  
CTGAATGACCTGGAAGCCTGTGTGATACAGGGGGTGGGGGTGACAGA  
GACTCCCCTGATGAAGGAGGACTCCATTCTGGCTGTGAGGAAATACT  
TCCAAAGAATCACTCTCTATCTGAAAGAGAAGAAATACAGCCCTTGT  
GCCTGGGAGGTTGTCAGAGCAGAAATCATGAGATCTTTTTCTTTGTCA  
ACAACTTGCAAGAAAGTTTAAGAAGTAAGGAATGAAAACCTGGTTCA  
ACATGGAAATGATTTTCATTGATTTCGTATGCCAGCTCACCTTTTTATG  
ATCTGCCATTTCAAAGACTCATGTTTCTGCTATGACCATGACACGATT  
TAAATCTTTTCAAATGTTTTTAGGAGTATTAATCAACATTGTATTGAG  
CTCTTAAGGCACTAGTCCCTTACAGAGGACCATGCTGACTGATCCATT  
ATCTATTTAAATATTTTAAAATATTATTTAATTTAACTATTTATAAAAC  
AACTTATTTTTGTTTCATATTATGTCATGTGCACCTTTGCACAGTGGTTA  
ATGTAATAAAAATGTGTTCTTTGTATTTGGTAAATTTATTTTGTGTTGTT  
CATTGAACTTTTGCTATGGAACCTTTGTACTTGTATTCTTTAAAATG  
AAATTCCAAGCCTAATTGTGCAACCTGATTACAGAATAACTGGTACA  
CTTCATTTGTCCATCAATATTATATTCAAGATATAAGTAAAAATAAAC  
TTTCTGTAAACCAAGTTGTATGTTGTACTCAAGATAACAGGGTGAACC  
TAACAAATACAATTCTGCTCTCTTGTGTATTTGATTTTGTATGAAAA  
AACTAAAAATGGTAATCATACTTAATTATCAGTTATGGTAAATGGT  
ATGAAGAGAAGAAGGAACG

279/498

FIG. 59B

Met Ala Leu Thr Phe Ala Leu Leu Val Ala Leu Leu Val Leu Ser Cys Lys Ser  
Ser Cys Ser Val Gly Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser Arg Arg Thr  
Leu Met Leu Leu Ala Gln Met Arg Arg Ile Ser Leu Phe Ser Cys Leu Lys Asp  
Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Gly Asn Gln Phe Gln Lys Ala  
Glu Thr Ile Pro Val Leu His Glu Met Ile Gln Gln Ile Phe Asn Leu Phe Ser Thr  
Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu  
Leu Tyr Gln Gln Leu Asn Asp Leu Glu Ala Cys Val Ile Gln Gly Val Gly Val  
Thr Glu Thr Pro Leu Met Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe  
Gln Arg Ile Thr Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val  
Val Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser Leu  
Arg Ser Lys Glu

FIG. 59C

ATGGCCCTCCTGTTCCCTCTACTGGCAGCCCTAGTGATGACCAGCTAT  
AGCCCTGTTGGATCTCTGGGCTGTGATCTGCCTCAGAACCATGGCCTA  
CTTAGCAGGAACACCTTGGTGCTTCTGCACCAAATGAGGAGAATCTCC  
CCTTTCTTGTGTCTCAAGGACAGAAGAGACTTCAGGTTCCCCCAGGAG  
ATGGTAAAAGGGAGCCAGTTGCAGAAGGCCCATGTCATGTCTGTCTCT  
CCATGAGATGCTGCAGCAGATCTTCAGCCTCTTCCACACAGAGCGCTC  
CTCTGCTGCCTGGAACATGACCCTCCTAGACCAACTCCCACTGGACT  
TCATCAGCAACTGCAACACCTGGAGACCTGCTTGCTGCAGGTAGTGG  
GAGAAGGAGAATCTGCTGGGGCAATTAGCAGCCCTGCACTGACCTTG  
AGGAGGTACTTCCAGGGAATCCGTGTCTACCTGAAAGAGAAGAAATA  
CAGCGACTGTGCCTGGGAAGTTGTCAGAATGGAAATCATGAAATCCT  
TGTTCTTATCAACAAACATGCAAGAAAGACTGAGAAGTAAAGATAGA  
GACCTGGGCTCATCTTGA

FIG. 59D

Met Ala Leu Leu Phe Pro Leu Leu Ala Ala Leu Val Met Thr Ser Tyr Ser Pro Val  
Gly Ser Leu Gly Cys Asp Leu Pro Gln Asn His Gly Leu Leu Ser Arg Asn Thr  
Leu Val Leu Leu His Gln Met Arg Arg Ile Ser Pro Phe Leu Cys Leu Lys Asp  
Arg Arg Asp Phe Arg Phe Pro Gln Glu Met Val Lys Gly Ser Gln Leu Gln Lys  
Ala His Val Met Ser Val Leu His Glu Met Leu Gln Gln Ile Phe Ser Leu Phe His  
Thr Glu Arg Ser Ser Ala Ala Trp Asn Met Thr Leu Leu Asp Gln Leu His Thr  
Gly Leu His Gln Gln Leu Gln His Leu Glu Thr Cys Leu Leu Gln Val Val Gly  
Glu Gly Glu Ser Ala Gly Ala Ile Ser Ser Pro Ala Leu Thr Leu Arg Arg Tyr Phe  
Gln Gly Ile Arg Val Tyr Leu Lys Glu Lys Lys Tyr Ser Asp Cys Ala Trp Glu Val  
Val Arg Met Glu Ile Met Lys Ser Leu Phe Leu Ser Thr Asn Met Gln Glu Arg  
Leu Arg Ser Lys Asp Arg Asp Leu Gly Ser Ser

280/498

FIG. 60A

ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCTTCTCC  
ACTACAGCTCTTTCCATGAGCTACAACCTTGCTTGGATTCTACAAAGA  
AGCAGCAATTTTCAGTGTGAGAAGCTCCTGTGGCAATTGAATGGGAG  
GCTTGAATATTGCCTCAAGGACAGGATGAACTTTGACATCCCTGAGG  
AGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCCGCATTGACC  
ATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCA  
TCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAA  
TGTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAAC  
TGGAGAAAGAAGATTTTACCAGGGGAAAACCTCATGAGCAGTCTGCAC  
CTGAAAAGATATTATGGGAGGATTCTGCATTACCTGAAGGCCAAGGA  
GTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATCCTAAGGA  
ACTTTTACTTCATTAAACAGACTTACAGGTTACCTCCGAAACTGAAGAT  
CTCCTAGCCTGTCCCTCTGGGACTGGACAATTGCTTCAAGCATTCTTC  
AACCAGCAGATGCTGTTTAAGTGACTGATGGCTAATGTACTGCAAAT  
GAAAGGACACTAGAAGATTTTGAAATTTTATTAAATTATGAGTTATT  
TTTATTTAT TTAAATTTTATTTTGAAAAATAAATTATTTTGGTGC

FIG. 60B

Met Thr Asn Lys Cys Leu Leu Gln Ile Ala Leu Leu Leu Cys Phe Ser Thr Thr Ala  
Leu Ser Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln  
Cys Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu Lys Asp  
Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys Gln Leu Gln Gln Phe Gln Lys Glu  
Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln Asn Ile Phe Ala Ile Phe Arg Gln  
Asp Ser Ser Ser Thr Gly Trp Asn Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val  
Tyr His Gln Ile Asn His Leu Lys Thr Val Leu Glu Glu Lys Leu Glu Lys Glu Asp  
Phe Thr Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys Arg Tyr Tyr Gly Arg Ile  
Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser His Cys Ala Trp Thr Ile Val Arg Val  
Glu Ile Leu Arg Asn Phe Tyr Phe Ile Asn Arg Leu Thr Gly Tyr Leu Arg Asn

281/498

FIG. 61A

ATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTGCTTGGGCTTCAG  
GGCTGCCTGGCTGCAGTCTTCGTAACCCAGGAGGAAGCCACGGCGT  
CCTGCACCGGCGCCGGCGCGCCAACGCGTTCCTGGAGGAGCTGCGGC  
CGGGCTCCCTGGAGAGGGAGTGCAAGGAGGAGCAGTGCTCCTTCGA  
GGAGGCCCGGGAGATCTTCAAGGACGCGGAGAGGACGAAGCTGTTC  
TGGATTTCTTACAGTGATGGGGACCAGTGTGCCTCAAGTCCATGCCA  
GAATGGGGGGCTCCTGCAAGGACCAGTCCAGTCCATATCTGCTTCT  
GCCTCCCTGCCTTTCGAGGGCCGGAAGTGTGAGACGCACAAGGATGAC  
CAGCTGATCTGTGTGAACGAGAACGGCGGCTGTGAGCAGTACTGCAG  
TGACCACACGGGCACCAAGCGTCTCTGTCGGTGCCACGAGGGGTACT  
CTCTGCTGGCAGACGGGGGTGTCCTGCACACCCACAGTTGAATATCCA  
TGTGGAAAAATACCTATTCTAGAAAAAAGAAATGCCAGCAAACCCCA  
AGGCCGAATTGTGGGGGGCAAGGTGTGCCCCAAAGGGGAGTGTCCA  
TGGCAGGTCCTGTTGTTGGTGAATGGAGCTCAGTTGTGTGGGGGGAC  
CCTGATCAACACCATCTGGGTGGTCTCCGCGGCCCCACTGTTTCGACAA  
AATCAAGAACTGGAGGAACCTGATCGCGGTGCTGGGCGAGCACGAC  
CTCAGCGAGCACGACGGGGATGAGCAGAGCCGGCGGGTGGCGCAGG  
TCATCATCCCCAGCACGTACGTCCCGGGCACCAACCAACGACATC  
GCGCTGCTCCGCCTGCACCAGCCCGTGGTCCTCACTGACCATGTGGTG  
CCCCTCTGCCTGCCCCGAACGGACGTTCTCTGAGAGGACGCTGGCCTTC  
GTGCGCTTCTCATTGGTTCAGCGGCTGGGGCCAGCTGCTGGACCGTGG  
CGCCACGGCCCTGGAGCTCATGGTGCTCAACGTGCCCCGGCTGATGA  
CCCAGGACTGCCTGCAGCAGTCACGGAAGGTGGGAGACTCCCCAAAT  
ATCACGGAGTACATGTTCTGTGCCGGCTACTCGGATGGCAGCAAGGA  
CTCCTGCAAGGGGGACAGTGGAGGCCACATGCCACCCACTACCGGG  
GCACGTGGTACCTGACGGGCATCGTCAGCTGGGGCCAGGGCTGCGCA  
ACCGTGGGCCACTTTGGGGTGTACACCAGGGTCTCCAGTACATCGA  
GTGGCTGCAAAAGCTCATGCGCTCAGAGCCACGCCCAGGAGTCCTCC  
TGCGAGCCCCATTCCC



282/498

FIG. 61B

Met Val Ser Gln Ala Leu Arg Leu Leu Cys Leu Leu Leu Gly Leu Gln Gly Cys  
Leu Ala Ala Val Phe Val Thr Gln Glu Glu Ala His Gly Val Leu His Arg Arg Arg  
Arg Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys  
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp Ala Glu Arg  
Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln Cys Ala Ser Ser Pro Cys  
Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro  
Ala Phe Glu Gly Arg Asn Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val  
Asn Glu Asn Gly Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg  
Ser Cys Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro  
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn Ala Ser Lys  
Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys Gly Glu Cys Pro Trp Gln  
Val Leu Leu Leu Val Asn Gly Ala Gln Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile  
Trp Val Val Ser Ala Ala His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile  
Ala Val Leu Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg  
Arg Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His Asp  
Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His Val Val Pro Leu  
Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu Ala Phe Val Arg Phe Ser  
Leu Val Ser Gly Trp Gly Gln Leu Leu Asp Arg Gly Ala Thr Ala Leu Glu Leu  
Met Val Leu Asn Val Pro Arg Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg  
Lys Val Gly Asp Ser Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp  
Gly Ser Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg  
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly  
His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys Leu Met  
Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe Pro

283/498

FIG. 62A

ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAAGCCTCATCAC  
CATCTGCCTTTTAGGATATCTACTCAGTGCTGAATGTACAGTTTTTCTT  
GATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAA  
TTCAGGTAAATTGGAAGAGTTTGTTCAGGGAACCTTGAGAGAGAAT  
GTATGGAAGAAAAGTGTAGTTTTGAAGAACCACGAGAAGTTTTTGAA  
AACACTGAAAAGACAACCTGAATTTTGAAGCAGTATGTTGATGGAGA  
TCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATG  
ACATTAATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGA  
ACTGTGAATTAGATGTAACATGTAACATTAAGAATGGCAGATGCGAG  
CAGTTTTGTAAAAATAGTGCTGATAACAAGGTGGTTTGCTCCTGTACT  
GAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTGAACCAGCAGT  
GCCATTTCCATGTGGAAGAGTTTCTGTTTCACAACTTCTAAGCTCAC  
CCGTGCTGAGGCTGTTTTTCCCTGATGTGGACTATGTAAATCCTACTGA  
AGCTGAAACCATTTTGGATAACATCACTCAAGGCACCCAATCATTTA  
ATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCAAACCAGGTCAA  
TTCCCTTGGCAGGTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGA  
GGCTCTATCGTTAATGAAAAATGGATTGTAACCTGCTGCCCCTGTGTT  
GAAACTGGTGTAAAATTACAGTTGTTCGCAGGTGAACATAATATTGA  
GGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAGCAATT  
ATTCCTCACCACAACCTACAATGCAGCTATTAATAAGTACAACCATGA  
CATTGCCCTTCTGGAACTGGACGAACCCTTAGTGCTAAACAGCTACG  
TTACACCTATTTGCATTGCTGACAAGGAATACACGAACATCTTCCTCA  
AATTTGGATCTGGCTATGTAAGTGGCTGGGCAAGAGTCTTCCACAAA  
GGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGAC  
CGAGCCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACAT  
GTTCTGTGCTGGCTTCCATGAAGGAGGTAGAGATTCATGTCAAGGAG  
ATAGTGGGGGACCCCATGTTACTGAAGTGAAGGGACCAGTTTCTTA  
ACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCCAAATA  
TGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAA  
AAACAAAGCTCACTTAATGAAAGATGGATTTCCAAGGTTAATTCATT  
GGAATTGAAAATTAACAG

284/498

FIG. 62B

Met Gln Arg Val Asn Met Ile Met Ala Glu Ser Pro Ser Leu Ile Thr Ile Cys Leu  
Leu Gly Tyr Leu Leu Ser Ala Glu Cys Thr Val Phe Leu Asp His Glu Asn Ala  
Asn Lys Ile Leu Asn Arg Pro Lys Arg Tyr Asn Ser Gly Lys Leu Glu Glu Phe  
Val Gln Gly Asn Leu Glu Arg Glu Cys Met Glu Glu Lys Cys Ser Phe Glu Glu  
Pro Arg Glu Val Phe Glu Asn Thr Glu Lys Thr Thr Glu Phe Trp Lys Gln Tyr  
Val Asp Gly Asp Gln Cys Glu Ser Asn Pro Cys Leu Asn Gly Gly Ser Cys Lys  
Asp Asp Ile Asn Ser Tyr Glu Cys Trp Cys Pro Phe Gly Phe Glu Gly Lys Asn  
Cys Glu Leu Asp Val Thr Cys Asn Ile Lys Asn Gly Arg Cys Glu Gln Phe Cys  
Lys Asn Ser Ala Asp Asn Lys Val Val Cys Ser Cys Thr Glu Gly Tyr Arg Leu  
Ala Glu Asn Gln Lys Ser Cys Glu Pro Ala Val Pro Phe Pro Cys Gly Arg Val Ser  
Val Ser Gln Thr Ser Lys Leu Thr Arg Ala Glu Ala Val Phe Pro Asp Val Asp Tyr  
Val Asn Pro Thr Glu Ala Glu Thr Ile Leu Asp Asn Ile Thr Gln Gly Thr Gln Ser  
Phe Asn Asp Phe Thr Arg Val Val Gly Gly Glu Asp Ala Lys Pro Gly Gln Phe  
Pro Trp Gln Val Val Leu Asn Gly Lys Val Asp Ala Phe Cys Gly Gly Ser Ile Val  
Asn Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Glu Thr Gly Val Lys Ile Thr  
Val Val Ala Gly Glu His Asn Ile Glu Glu Thr Glu His Thr Glu Gln Lys Arg Asn  
Val Ile Arg Ala Ile Ile Pro His His Asn Tyr Asn Ala Ala Ile Asn Lys Tyr Asn  
His Asp Ile Ala Leu Leu Glu Leu Asp Glu Pro Leu Val Leu Asn Ser Tyr Val Thr  
Pro Ile Cys Ile Ala Asp Lys Glu Tyr Thr Asn Ile Phe Leu Lys Phe Gly Ser Gly  
Tyr Val Ser Gly Trp Ala Arg Val Phe His Lys Gly Arg Ser Ala Leu Val Leu Gln  
Tyr Leu Arg Val Pro Leu Val Asp Arg Ala Thr Cys Leu Arg Ser Thr Lys Phe  
Thr Ile Tyr Asn Asn Met Phe Cys Ala Gly Phe His Glu Gly Gly Arg Asp Ser  
Cys Gln Gly Asp Ser Gly Gly Pro His Val Thr Glu Val Glu Gly Thr Ser Phe Leu  
Thr Gly Ile Ile Ser Trp Gly Glu Glu Cys Ala Met Lys Gly Lys Tyr Gly Ile Tyr  
Thr Lys Val Ser Arg Tyr Val Asn Trp Ile Lys Glu Lys Thr Lys Leu Thr

285/498

FIG. 63A

ATGGATTACTACAGAAAATATGCAGCTATCTTTCTGGTCACATTGTCCG  
GTGTTTCTGCATGTTCTCCATTCCGCTCCTGATGTGCAGGATTGCCCA  
GAATGCACGCTACAGGAAAACCCATTCTTCTCCCAGCCGGGTGCCCC  
AATACTTCAGTGCATGGGCTGCTGCTTCTCTAGAGCATATCCCCTCC  
ACTAAGGTCCAAGAAGACGATGTTGGTCCAAAAGAACGTCACCTCAG  
AGTCCACTTGCTGTGTAGCTAAATCATATAACAGGGTCACAGTAATG  
GGGGGTTTCAAAGTGGAGAACCACACGGCGTGCCACTGCAGTACTTG  
TTATTATCACAAATCTTAAATGTTTTACCAAGTGCTGTCTTGATGACT  
GCTGATTTTCTGGAATGGAAAATTAAGTTGTTTAGTGTTTATGGCTTT  
GTGAGATAAACTCTCCTTTTCCTTACCATACCACTTTGACACGCTTC  
AAGGATATACTGCAGCTTTACTGCCTTCCTCCTTATCCTACAGTACAA  
TCAGCAGTCTAGTTCTTTTCATTTGGAATGAATACAGCATTAAAGCTTG  
TTCCACTGCAAATAAAGCCTTTTAAATCATC

FIG. 63B

Met Asp Tyr Tyr Arg Lys Tyr Ala Ala Ile Phe Leu Val Thr Leu Ser Val Phe Leu  
His Val Leu His Ser Ala Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu  
Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe  
Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn  
Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met  
Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His  
Lys Ser

286/498

FIG. 63C

ATGAAGACACTCCAGTTTTTCTTCCTTTTCTGTTGCTGGAAAGCAATC  
TGCTGCAATAGCTGTGAGCTGACCAACATCACCATTGCAATAGAGAA  
AGAAGAATGTCGTTTCTGCATAAGCATCAACACCACTTGGTGTGCTG  
GCTACTGCTACACCAGGGATCTGGTGTATAAGGACCCAGCCAGGCCC  
AAAATCCAGAAAACATGTACCTTCAAGGAAGTGGTATATGAAACAGT  
GAGAGTGCCCGGCTGTGCTCACCATGCAGATTCCTTGTATACATACCC  
AGTGGCCACCCAGTGTCACTGTGGCAAGTGTGACAGCGACAGCACTG  
ATTGTACTGTGCGAGGCCTGGGGCCCAGCTACTGCTCCTTTGGTGAAA  
TGAAAGAATAA

FIG. 63D

Met Lys Thr Leu Gln Phe Phe Phe Leu Phe Cys Cys Trp Lys Ala Ile Cys Cys  
Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys Glu Glu Cys Arg Phe  
Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly Tyr Cys Tyr Thr Arg Asp Leu Val  
Tyr Lys Asp Pro Ala Arg Pro Lys Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val  
Tyr Glu Thr Val Arg Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr  
Pro Val Ala Thr Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys  
Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu Met Lys Glu

287/498

FIG. 64A

CCCGGAGCCGGACCGGGGCCACCGCGCCCGCTCTGCTCCGACACCGC  
GCCCCCTGGACAGCCGCCCTCTCCTCCAGGCCCGTGGGGCTGGCCCT  
GCACCGCCGAGCTTCCCGGGATGAGGGCCCCCGGTGTGGTCACCCGG  
CGCGCCCCAGGTCGCTGAGGGACCCCGGCCAGGCGCGGAGATGGGG  
GTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCTGTGC  
CTCCCTCTGGGCCTCCAGTCCTGGGCGCCCCACCACGCCTCATCTGT  
GACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCG  
AGAATATCACGACGGGCTGTGCTGAACACTGCAGCTTGAATGAGAAT  
ATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAGGAT  
GGAGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAGGGCCTGGCCCTG  
CTGTGCGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCAACTCTTCC  
CAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTCAGTGG  
CCTTCGCAGCCTCACCCTCTGCTTCGGGCTCTGCGAGCCCAGAAGG  
AAGCCATCTCCCCTCCAGATGCGGCCTCAGCTGCTCCACTCCGAACA  
ATCACTGCTGACACTTTCCGCAAACCTCTTCCGAGTCTACTCCAATTTC  
CTCCGGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTGCAGGACAG  
GGGACAGATGACCAGGTGTGTCCACCTGGGCATATCCACCACCTCCC  
TCACCAACATTGCTTGTGCCACACCCTCCCCCGCCACTCCTGAACCCC  
GTCGAGGGGCTCTCAGCTCAGCGCCAGCCTGTCCCATGGACACTCCA  
GTGCCAGCAATGACATCTCAGGGGCCAGAGGAACTGTCCAGAGAGC  
AACTCTGAGATCTAAGGATGTCACAGGGGCCAACTTGAGGGCCCAGAG  
CAGGAAGCATTGAGAGAGCAGCTTTAAACTCAGGGACAGAGCCATG  
CTGGGAAGACGCCTGAGCTCACTCGGCACCCTGCAAAATTTGATGCC  
AGGACACGCTTTGGAGGCGATTTACCTGTTTTCGCACCTACCATCAGG  
GACAGGATGACCTGGAGAACTTAGGTGGCAAGCTGTGACTTCTCCAG  
GTCTCACGGGCATGGGCACTCCCTTGGTGGCAAGAGCCCCCTTGACA  
CCGGGGTGGTGGGAACCATGAAGACAGGATGGGGGCTGGCCTCTGG  
CTCTCATGGGGTCCAAGTTTTGTGTATTCTTCAACCTCATTGACAAGA  
ACTGAAACCACCAAAAAAAAAAAAAA

288/498

FIG. 64B

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu Leu Ser  
Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu Ile Cys Asp Ser  
Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr  
Gly Cys Ala Glu His Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys  
Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val  
Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu  
Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser  
Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Arg Ala Gln Lys Glu Ala Ile  
Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe  
Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr  
Thr Gly Glu Ala Cys Arg Thr Gly Asp Arg

FIG. 65

Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala  
Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu Asn  
Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly  
Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg  
Gly Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val  
Asp Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly  
Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr  
Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg Gly  
Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp

289/498

FIG. 66A

ATGTGGCTGCAGAGCCTGCTGCTCTTGGGCACTGTGGCCTGCAGCAT  
CTCTGCACCCGCCCCGCTCGCCCAGCCCCAGCACGCAGCCCTGGGAGC  
ATGTGAATGCCATCCAGGAGGCCCGGCGTCTCCTGAACCTGAGTAGA  
GAACTGCTGCTGAGATGAATGAAACAGTAGAAGTCATCTCAGAAAT  
GTTTGACCTCCAGGAGCCGACCTGCCTACAGACCCGCCTGGAGCTGT  
ACAAGCAGGGCCTGCGGGGCAGCCTACCAAGCTCAAGGGCCCCTTG  
ACCATGATGGCCAGCCACTACAAGCAGCACTGCCCTCCAACCCCGGA  
AACTTCCTGTGCAACCCAGATTATCACCTTTGAAAGTTTCAAAGAGA  
ACCTGAAGGACTTTCTGCTTGTCATCCCCTTTGACTGCTGGGAGCCAG  
TCCAGGAGTGA

FIG. 66B

Met Trp Leu Gln Ser Leu Leu Leu Leu Gly Thr Val Ala Cys Ser Ile Ser Ala Pro  
Ala Arg Ser Pro Ser Pro Ser Thr Gln Pro Trp Glu His Val Asn Ala Ile Gln Glu  
Ala Arg Arg Leu Leu Asn Leu Ser Arg Asp Thr Ala Ala Glu Met Asn Glu Thr  
Val Glu Val Ile Ser Glu Met Phe Asp Leu Gln Glu Pro Thr Cys Leu Gln Thr Arg  
Leu Glu Leu Tyr Lys Gln Gly Leu Arg Gly Ser Leu Thr Lys Leu Lys Gly Pro  
Leu Thr Met Met Ala Ser His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Thr Ser  
Cys Ala Thr Gln Ile Ile Thr Phe Glu Ser Phe Lys Glu Asn Leu Lys Asp Phe Leu  
Leu Val Ile Pro Phe Asp Cys Trp Glu Pro Val Gln Glu



290/498

FIG. 67A

ATGAAATATACAAGTTATATCTTGGCTTTTCAGCTCTGCATCGTTTTG  
GGTTCTCTTGGCTGTTACTGCCAGGACCCATATGTAAAAGAAGCAGA  
AAACCTTAAGAAATATTTTAATGCAGGTCATTCAGATGTAGCGGATA  
ATGGAACTCTTTTCTTAGGCATTTTGAAGAATTGGAAAGAGGAGAGT  
GACAGAAAAATAATGCAGAGCCAAATTGTCTCCTTTTACTTCAAAC  
TTTTAAAAACTTTTAAAGATGACCAGAGCATCCAAAAGAGTGTGGAGA  
CCATCAAGGAAGACATGAATGTCAAGTTTTTCAATAGCAACAAAAAG  
AAACGAGATGACTTCGAAAAGCTGACTAATTATTCGGTAACTGACTT  
GAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGATGGCTG  
AACTGTCCGCCAGCAGCTAAAACAGGGAAGCGAAAAAGGAGTCAGAT  
GCTGTTTCGAGGTCGAAGAGCATCCCAGTAA

FIG. 67B

Met Lys Tyr Thr Ser Tyr Ile Leu Ala Phe Gln Leu Cys Ile Val Leu Gly Ser Leu  
Gly Cys Tyr Cys Gln Asp Pro Tyr Val Lys Glu Ala Glu Asn Leu Lys Lys Tyr  
Phe Asn Ala Gly His Ser Asp Val Ala Asp Asn Gly Thr Leu Phe Leu Gly Ile  
Leu Lys Asn Trp Lys Glu Glu Ser Asp Arg Lys Ile Met Gln Ser Gln Ile Val Ser  
Phe Tyr Phe Lys Leu Phe Lys Asn Phe Lys Asp Asp Gln Ser Ile Gln Lys Ser Val  
Glu Thr Ile Lys Glu Asp Met Asn Val Lys Phe Phe Asn Ser Asn Lys Lys Lys  
Arg Asp Asp Phe Glu Lys Leu Thr Asn Tyr Ser Val Thr Asp Leu Asn Val Gln  
Arg Lys Ala Ile His Glu Leu Ile Gln Val Met Ala Glu Leu Ser Pro Ala Ala Lys  
Thr Gly Lys Arg Lys Arg Ser Gln Met Leu Phe Arg Gly Arg Arg Ala Ser Gln

291/498

FIG. 68A

CTGGGACAGTGAATCGACAATGCCGTCTTCTGTCTCGTGGGGCATCCT  
CCTGCTGGCAGGCCTGTGCTGCCTGGTCCCTGTCTCCCTGGCTGAGGA  
TCCCCAGGGAGATGCTGCCCAGAAGACAGATACATCCCACCATGATC  
AGGATCACCCAACCTTCAACAAGATCACCCCCAACCTGGCTGAGTTC  
GCCTTCAGCCTATACCGCCAGCTGGCACACCAGTCCAACAGCACCAA  
TATCTTCTTCTCCCCAGTGAGCATCGCTACAGCCTTTGCAATGCTCTC  
CCTGGGGACCAAGGCTGACACTCACGATGAAATCCTGGAGGGCCTGA  
ATTTCAACCTCACGGAGATTCCGGAGGCTCAGATCCATGAAGGCTTC  
CAGGAACCTCCTCCGTACCCTCAACCAGCCAGACAGCCAGCTCCAGCT  
GACCACCGGCAATGGCCTGTTCTCAGCGAGGGCCTGAAGCTAGTGG  
ATAAGTTTTTGGAGGATGTTAAAAAGTTGTACCACTCAGAAGCCTTC  
ACTGTCAACTTCGGGGACACCGAAGAGGCCAAGAAACAGATCAACG  
ATTACGTGGAGAAGGGTACTCAAGGGGAAAATTGTGGATTTGGTCAAG  
GAGCTTGACAGAGACACAGTTTTTGTCTCTGGTGAATTACATCTTCTTT  
AAAGGCAAATGGGAGAGACCCTTTGAAGTCAAGGACACCGAGGAAG  
AGGACTTCCACGTGGACCAGGTGACCACCGTGAAGGTGCCTATGATG  
AAGCGTTTAGGCATGTTTAAACATCCAGCACTGTAAGAAGCTGTCCAG  
CTGGGTGCTGCTGATGAAATACCTGGGCAATGCCACCGCCATCTTCT  
TCCTGCCTGATGAGGGGAAACTACAGCACCTGGAAAATGAACTCACC  
CACGATATCATCACCAAGTTCCTGGAAAATGAAGACAGAAGGTCTGC  
CAGCTTACATTTACCCAAACTGTCCATTACTGGAACCTATGATCTGAA  
GAGCGTCCTGGGTCAACTGGGCATCACTAAGGTCTTCAGCAATGGGG  
CTGACCTCTCCGGGGTACAGAGGAGGCACCCCTGAAGCTCTCCAAG  
GCCGTGCATAAGGCTGTGCTGACCATCGACGAGAAAGGGACTGAAGC  
TGCTGGGGCCATGTTTTTAGAGGCCATACCCATGTCTATCCCCCCCCGA  
GGTCAAGTTCAACAAACCCTTTGTCTTCTTAATGATTGAACAAAATAC  
CAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAATCCCACCCAAAAAT  
AACTGCCTCTCGCTCCTCAACCCCTCCCCTCCATCCCTGGCCCCCTCC  
CTGGATGACATTAAAGAAGGGTTGAGCTGG

292/498

FIG. 68B

Met Pro Ser Ser Val Ser Trp Gly Ile Leu Leu Leu Ala Gly Leu Cys Cys Leu Val  
Pro Val Ser Leu Ala Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser  
His His Asp Gln Asp His Pro Thr Phe Asn Lys Ile Thr Pro Asn Leu Ala Glu Phe  
Ala Phe Ser Leu Tyr Arg Gln Leu Ala His Gln Ser Asn Ser Thr Asn Ile Phe Phe  
Ser Pro Val Ser Ile Ala Thr Ala Phe Ala Met Leu Ser Leu Gly Thr Lys Ala Asp  
Thr His Asp Glu Ile Leu Glu Gly Leu Asn Phe Asn Leu Thr Glu Ile Pro Glu Ala  
Gln Ile His Glu Gly Phe Gln Glu Leu Leu Arg Thr Leu Asn Gln Pro Asp Ser Gln  
Leu Gln Leu Thr Thr Gly Asn Gly Leu Phe Leu Ser Glu Gly Leu Lys Leu Val  
Asp Lys Phe Leu Glu Asp Val Lys Lys Leu Tyr His Ser Glu Ala Phe Thr Val  
Asn Phe Gly Asp Thr Glu Glu Ala Lys Lys Gln Ile Asn Asp Tyr Val Glu Lys  
Gly Thr Gln Gly Lys Ile Val Asp Leu Val Lys Glu Leu Asp Arg Asp Thr Val  
Phe Ala Leu Val Asn Tyr Ile Phe Phe Lys Gly Lys Trp Glu Arg Pro Phe Glu Val  
Lys Asp Thr Glu Glu Glu Asp Phe His Val Asp Gln Val Thr Thr Val Lys Val  
Pro Met Met Lys Arg Leu Gly Met Phe Asn Ile Gln His Cys Lys Lys Leu Ser  
Ser Trp Val Leu Leu Met Lys Tyr Leu Gly Asn Ala Thr Ala Ile Phe Phe Leu Pro  
Asp Glu Gly Lys Leu Gln His Leu Glu Asn Glu Leu Thr His Asp Ile Ile Thr Lys  
Phe Leu Glu Asn Glu Asp Arg Arg Ser Ala Ser Leu His Leu Pro Lys Leu Ser Ile  
Thr Gly Thr Tyr Asp Leu Lys Ser Val Leu Gly Gln Leu Gly Ile Thr Lys Val Phe  
Ser Asn Gly Ala Asp Leu Ser Gly Val Thr Glu Glu Ala Pro Leu Lys Leu Ser Lys  
Ala Val His Lys Ala Val Leu Thr Ile Asp Glu Lys Gly Thr Glu Ala Ala Gly Ala  
Met Phe Leu Glu Ala Ile Pro Met Ser Ile Pro Pro Glu Val Lys Phe Asn Lys Pro  
Phe Val Phe Leu Met Ile Glu Gln Asn Thr Lys Ser Pro Leu Phe Met Gly Lys Val  
Val Asn Pro Thr Gln Lys

293/498

FIG. 69A-1

GCTAACCTAGTGCCTATAGCTAAGGCAGGTACCTGCATCCTTGTTTTT  
GTTTAGTGGATCCTCTATCCTTCAGAGACTCTGGAACCCCTGTGGTCT  
TCTCTTCATCTAATGACCCTGAGGGGATGGAGTTTTCAAGTCCTTCCA  
GAGAGGAATGTCCCAAGCCTTTGAGTAGGGTAAGCATCATGGCTGGC  
AGCCTCACAGGTTTGCTTCTACTTCAGGCAGTGTCGTGGGCATCAGGT  
GCCCGCCCCTGCATCCCTAAAAGCTTCGGCTACAGCTCGGTGGTGTGT  
GTCTGCAATGCCACATACTGTGACTCCTTTGACCCCCCGACCTTTCCT  
GCCCTTGGTACCTTCAGCCGCTATGAGAGTACACGCAGTGGGCGACG  
GATGGAGCTGAGTATGGGGCCCATCCAGGCTAATCACACGGGCACAG  
GCCTGCTACTGACCCTGCAGCCAGAACAGAAGTTCCAGAAAGTGAAG  
GGATTTGGAGGGGGCCATGACAGATGCTGCTGCTCTCAACATCCTTGCC  
CTGTACCCCCCTGCCAAAATTTGCTACTTAAATCGTACTTCTCTGAA  
GAAGGAATCGGATATAACATCATCCGGGTACCCATGGCCAGCTGTGA  
CTTCTCCATCCGCACCTACACCTATGCAGACACCCCTGATGATTTCCA  
GTTGCACAACCTTCAGCCTCCCAGAGGAAGATACCAAGCTCAAGATAC  
CCCTGATTCACCGAGCCCTGCAGTTGGCCCAGCGTCCCGTTTCACTCC  
TTGCCAGCCCCTGGACATCACCCACTTGGCTCAAGACCAATGGAGCG  
GTGAATGGGAAGGGGTCACTCAAGGGACAGCCCGGAGACATCTACC  
ACCAGACCTGGGCCAGATACTTTGTGAAGTTCCTGGATGCCTATGCTG  
AGCACAAGTTACAGTTCTGGGCAGTGACAGCTGAAAATGAGCCTTCT  
GCTGGGCTGTTGAGTGGATACCCCTTCCAGTGCCTGGGCTTCACCCCT  
GAACATCAGCGAGACTTCATTGCCCGTGACCTAGGTCCTACCCTCGCC  
AACAGTACTCACCACAATGTCCGCCTACTCATGCTGGATGACCAACGC  
TTGCTGCTGCCCCACTGGGCAAAGGTGGTACTGACAGACCCAGAAGC  
AGCTAAATATGTTTCATGGCATTGCTGTACATTGGTACCTGGACTTTCT  
GGCTCCAGCCAAAGCCACCCTAGGGGAGACACACCGCCTGTTCCCCA  
ACACCATGCTCTTTGCCTCAGAGGCCTGTGTGGGCTCCAAGTTCTGGG  
AGCAGAGTGTGCGGCTAGGCTCCTGGGATCGAGGGATGCAGTACAGC  
CACAGCATCATCACGAACCTCCTGTACCATGTGGTCGGCTGGACCGAC  
TGGAACCTTGCCCTGAACCCCGAAGGAGGACCCAATTGGGTGCGTAA  
CTTTGTCGACAGTCCCATCATTGTAGACATCACCAAGGACACGTTTTA  
CAAACAGCCCATGTTCTACCACCTTGGCCACTTCAGCAAGTTCATTCC  
TGAGGGCTCCCAGAGAGTGGGGCTGGTTGCCAGTCAGAAGAACGACC  
TGGACGCAGTGGCACTGATGCATCCCGATGGCTCTGCTGTTGTGGTCG  
TGCTAAACCGCTCCTCTAAGGATGTGCCTCTTACCATCAAGGATCCTG  
CTGTGGGCTTCTGGAGACAATCTCACCTGGCTACTCCATTACACCT  
ACCTGTGGCATCGCCAGTGATGGAGCAGATACTCAAGGAGGCACTGG  
GCTCAGCCTGGGCATTAAAGGGACAGAGTCAGCTCACACGCTGTCTG  
TGACTAAAGAGGGGCACAGCAGGGCCAGTGTGAGCTTACAGCGACGT

294/498

FIG. 69A-2

AAGCCCAGGGGCAATGGTTTGGGTGACTCACTTCCCCCTCTAGGTGGT  
GCCCAGGGCTGGAGGCCCTAGAAAAAGATCAGTAAGCCCCAGTGTC  
CCCCAGCCCCCATGCTTATGTGAACATGCGCTGTGTGCTGCTTGCTT  
TGGAAACT

FIG. 69B

Met Glu Phe Ser Ser Pro Ser Arg Glu Glu Cys Pro Lys Pro Leu Ser Arg Val Ser  
Ile Met Ala Gly Ser Leu Thr Gly Leu Leu Leu Leu Gln Ala Val Ser Trp Ala Ser  
Gly Ala Arg Pro Cys Ile Pro Lys Ser Phe Gly Tyr Ser Ser Val Val Cys Val Cys  
Asn Ala Thr Tyr Cys Asp Ser Phe Asp Pro Pro Thr Phe Pro Ala Leu Gly Thr  
Phe Ser Arg Tyr Glu Ser Thr Arg Ser Gly Arg Arg Met Glu Leu Ser Met Gly  
Pro Ile Gln Ala Asn His Thr Gly Thr Gly Leu Leu Leu Thr Leu Gln Pro Glu Gln  
Lys Phe Gln Lys Val Lys Gly Phe Gly Gly Ala Met Thr Asp Ala Ala Ala Leu  
Asn Ile Leu Ala Leu Ser Pro Pro Ala Gln Asn Leu Leu Leu Lys Ser Tyr Phe Ser  
Glu Glu Gly Ile Gly Tyr Asn Ile Ile Arg Val Pro Met Ala Ser Cys Asp Phe Ser  
Ile Arg Thr Tyr Thr Tyr Ala Asp Thr Pro Asp Asp Phe Gln Leu His Asn Phe Ser  
Leu Pro Glu Glu Asp Thr Lys Leu Lys Ile Pro Leu Ile His Arg Ala Leu Gln Leu  
Ala Gln Arg Pro Val Ser Leu Leu Ala Ser Pro Trp Thr Ser Pro Thr Trp Leu Lys  
Thr Asn Gly Ala Val Asn Gly Lys Gly Ser Leu Lys Gly Gln Pro Gly Asp Ile  
Tyr His Gln Thr Trp Ala Arg Tyr Phe Val Lys Phe Leu Asp Ala Tyr Ala Glu  
His Lys Leu Gln Phe Trp Ala Val Thr Ala Glu Asn Glu Pro Ser Ala Gly Leu  
Leu Ser Gly Tyr Pro Phe Gln Cys Leu Gly Phe Thr Pro Glu His Gln Arg Asp  
Phe Ile Ala Arg Asp Leu Gly Pro Thr Leu Ala Asn Ser Thr His His Asn Val Arg  
Leu Leu Met Leu Asp Asp Gln Arg Leu Leu Leu Pro His Trp Ala Lys Val Val  
Leu Thr Asp Pro Glu Ala Ala Lys Tyr Val His Gly Ile Ala Val His Trp Tyr Leu  
Asp Phe Leu Ala Pro Ala Lys Ala Thr Leu Gly Glu Thr His Arg Leu Phe Pro  
Asn Thr Met Leu Phe Ala Ser Glu Ala Cys Val Gly Ser Lys Phe Trp Glu Gln Ser  
Val Arg Leu Gly Ser Trp Asp Arg Gly Met Gln Tyr Ser His Ser Ile Ile Thr Asn  
Leu Leu Tyr His Val Val Gly Trp Thr Asp Trp Asn Leu Ala Leu Asn Pro Glu  
Gly Gly Pro Asn Trp Val Arg Asn Phe Val Asp Ser Pro Ile Ile Val Asp Ile Thr  
Lys Asp Thr Phe Tyr Lys Gln Pro Met Phe Tyr His Leu Gly His Phe Ser Lys  
Phe Ile Pro Glu Gly Ser Gln Arg Val Gly Leu Val Ala Ser Gln Lys Asn Asp Leu  
Asp Ala Val Ala Leu Met His Pro Asp Gly Ser Ala Val Val Val Val Leu Asn  
Arg Ser Ser Lys Asp Val Pro Leu Thr Ile Lys Asp Pro Ala Val Gly Phe Leu Glu  
Thr Ile Ser Pro Gly Tyr Ser Ile His Thr Tyr Leu Trp His Arg Gln

295/498

FIG. 70A

ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGG  
AGCAGTCTTCGTTTCGCCAGCCAGGAAATCCATGCCCCGATTCAGAA  
GAGGAGCCAGATCTTACCAAGTGATCTGCAGAGATGAAAAAACGCA  
GATGATATAACCAGCAACATCAGTCATGGCTGCGCCCTGTGCTCAGAA  
GCAACCGGGTGGAAATATTGCTGGTGCAACAGTGGCAGGGGCACAGTGC  
CACTCAGTGCCTGTCAAAAGTTGAGCGAGCCAAGGTGTTTCAACGG  
GGGCACCTGCCAGCAGGCCCTGTACTTCTCAGATTTTCGTGTGCCAGTG  
CCCCGAAGGATTTGCTGGGAAGTGCTGTGAAATAGATACCAGGGCCA  
CGTGCTACGAGGACCAGGGCATCAGCTACAGGGGGCACGTGGAGCAC  
AGCGGAGAGTGGCGCCGAGTGACCAACTGGAACAGCAGCGCGTTG  
GCCAGAAGCCCTACAGCGGGCGGAGGCCAGACGCCATCAGGCTGG  
GCCTGGGGAACCACAACACTACTGCAGAAACCAGATCGAGACTCAA  
GCCCTGGTGCTACGTCTTTAAGGCGGGGAAGTACAGCTCAGAGTTCT  
GCAGCACCCCTGCCTGCTCTGAGGGAAACAGTGACTGCTACTTTGGG  
AATGGGTCAGCCTACCGTGGCACGCACAGCCTCACCGAGTCGGGTGC  
CTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGGCAAGGTTTACAC  
AGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATAATT  
ACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTG  
AAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTC  
CACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAG  
GAGGGCTCTTCGCCGACATCGCCTCCACCCCTGGCAGGCTGCCATCT  
TTGCCAAGCACAGGAGGTCGCCGGGAGAGCGGTTCCCTGTGCGGGGGC  
ATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCCACTGCTTCCAG  
GAGAGGTTTCCGCCCCACCACTGACGGTGATCTTGGGCAGAACATA  
CCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAA  
TACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACAT  
TGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGA  
GCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTG  
CCGGA CTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGC  
CTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACT  
GTACCCATCCAGCCGCTGCACATCACAAACATTTACTTAACAGAACAG  
TCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCC  
CAGGCAAACCTTGACGACGCCTGCCAGGGCGATTTCGGGAGGCCCCCT  
GGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCT  
GGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACCAAG  
GTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCGTGACC  
AGGAACACCCGACTCCTCAAAAGCAAATGAGATCC

296/498

FIG. 70B

Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu Leu Cys Gly Ala Val  
Phe Val Ser Pro Ser Gln Glu Ile His Ala Arg Phe Arg Arg Gly Ala Arg Ser Tyr  
Gln Val Ile Cys Arg Asp Glu Lys Thr Gln Met Ile Tyr Gln Gln His Gln Ser Trp  
Leu Arg Pro Val Leu Arg Ser Asn Arg Val Glu Tyr Cys Trp Cys Asn Ser Gly  
Arg Ala Gln Cys His Ser Val Pro Val Lys Ser Cys Ser Glu Pro Arg Cys Phe Asn  
Gly Gly Thr Cys Gln Gln Ala Leu Tyr Phe Ser Asp Phe Val Cys Gln Cys Pro  
Glu Gly Phe Ala Gly Lys Cys Cys Glu Ile Asp Thr Arg Ala Thr Cys Tyr Glu  
Asp Gln Gly Ile Ser Tyr Arg Gly Thr Trp Ser Thr Ala Glu Ser Gly Ala Glu Cys  
Thr Asn Trp Asn Ser Ser Ala Leu Ala Gln Lys Pro Tyr Ser Gly Arg Arg Pro Asp  
Ala Ile Arg Leu Gly Leu Gly Asn His Asn Tyr Cys Arg Asn Pro Asp Arg Asp  
Ser Lys Pro Trp Cys Tyr Val Phe Lys Ala Gly Lys Tyr Ser Ser Glu Phe Cys Ser  
Thr Pro Ala Cys Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr  
Arg Gly Thr His Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp Asn Ser Met  
Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu  
Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His  
Val Leu Lys Asn Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser  
Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe  
Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser  
Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser  
Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile Leu Gly  
Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys  
Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu  
Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg  
Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu  
Leu Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys  
Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn  
Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro  
Gln Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys  
Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly  
Gln Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg  
Asp Asn Met Arg Pro

297/498

FIG. 71A

ATCACTCTCTTTAATCACTACTCACATTAACCTCAACTCCTGCCACAA  
TGTACAGGATGCAACTCCTGTCTTGCATTGCACTAATTCTTGCACCTG  
TCACAAACAGTGCACCTACTTCAAGTTCGACAAAGAAAACAAAGAAA  
ACACAGCTACAACCTGGAGCATTACTGCTGGATTACAGATGATTTTG  
AATGGAATTAATAATTACAAGAATCCCAAACCTCACCAGGATGCTCAC  
ATTTAAGTTTTACATGCCCAAGAAGGCCACAGAAGTGAACAGCTTC  
AGTGTCTAGAAGAAGAAGTCAAACCTCTGGAGGAAGTGCTGAATTTA  
GCTCAAAGCAAAAACCTTCACTTAAGACCCAGGGACTTAATCAGCAA  
TATCAACGTAATAGTTCTGGAAGTAAAGGGATCTGAAACAACATTCA  
TGTGTGAATATGCAGATGAGACAGCAACCATTGTAGAATTTCTGAAC  
AGATGGATTACCTTTTGTCAAAGCATCATCTCAACACTAAGTGTATAA  
TTAAGTGCTTCCCACTTAAACATATCAGGCCTTCTATTTATTTATTTA  
AATATTTAAATTTTATATTTATTGTTGAATGTATGGTTGCTACCTATTG  
TAACTATTATTCTTAATCTTAAAGTATAAATATGGATCTTTTATGAT  
TCTTTTGTGAAGCCCTAGGGGCTCTAAAGTGGTTTACCTTATTTATCC  
CAAAAATATTTATTATTATGTTGAATGTAAATATAGTATCTATGTAG  
ATTGGTTAGTAAAGTATTTAATAAATTTGATAAATATAAAAAAAAAA  
AAACAAAAAAAAAAAA

FIG. 71B

Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ile Leu Ala Leu Val Thr Asn  
Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Lys Lys Thr Gln Leu Gln Leu Glu  
His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn  
Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr  
Glu Leu Lys Gln Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val  
Leu Asn Leu Ala Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser  
Asn Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu  
Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys  
Gln Ser Ile Ile Ser Thr Leu Thr



298/498

FIG. 72A-1

ATGCAAATAGAGCTCTCCACCTGCTTCTTTCTGTGCCTTTTGCGATTCT  
GCTTTAGTGCCACCAGAAGATACTACCTGGGTGCAGTGGAAGTGTCA  
TGGGACTATATGCAAAGTGATCTCGGTGAGCTGCCTGTGGACGCAAG  
ATTCCTCCTAGAGTGCCAAAATCTTTTCCATTCAACACCTCAGTCGT  
GTACAAAAAGACTCTGTTTGTAGAATTCACGGATCACCTTTTCAACAT  
CGCTAAGCCAAGGCCACCCTGGATGGGTCTGCTAGGTCCTACCATCC  
AGGCTGAGGTTTATGATACAGTGGTCATTACACTTAAGAACATGGCT  
TCCCATCCTGTCAGTCTTCATGCTGTTGGTGTATCCTACTGGAAAGCT  
TCTGAGGGAGCTGAATATGATGATCAGACCAGTCAAAGGGAGAAAG  
AAGATGATAAAGTCTTCCCTGGTGGAAGCCATACATATGTCTGGCAG  
GTCCTGAAAGAGAATGGTCCAATGGCCTCTGACCCACTGTGCCTTAC  
CTACTCATATCTTTCTCATGTGGACCTGGTAAAAGACTTGAATTCAGG  
CCTCATTGGAGCCCTACTAGTATGTAGAGAAGGGAGTCTGGCCAAGG  
AAAAGACACAGACCTTGCACAAATTTATACTACTTTTTTGCTGTATTG  
ATGAAGGGAAAAGTTGGCACTCAGAAACAAAGAACTCCTTGATGCA  
GGATAGGGATGCTGCATCTGCTCGGGCCTGGCCTAAAATGCACACAG  
TCAATGGTTATGTAAACAGGTCTCTGCCAGGTCTGATTGGATGCCACA  
GGAAATCAGTCTATTGGCATGTGATTGGAATGGGCACCACTCCTGAA  
GTGCACTCAATATTCCTCGAAGGTCACACATTTCTTGAGGAACCAT  
CGCCAGGCGTCCTTGGAATCTCGCCAATAACTTTCTTACTGCTCAA  
ACACTCTTGATGGACCTTGGACAGTTTCTACTGTTTTGTCATATCTCTT  
CCCACCAACATGATGGCATGGAAGCTTATGTCAAAGTAGACAGCTGT  
CCAGAGGAACCCCACTACGAATGAAAAATAATGAAGAAGCGGAAG  
ACTATGATGATGATCTTACTGATTCTGAAATGGATGTGGTCAGGTTTG  
ATGATGACAACTCTCCTTCCTTTATCCAAATTCGCTCAGTTGCCAAGA  
AGCATCCTAAAACCTTGGGTACATTACATTGCTGCTGAAGAGGAGGAC  
TGGGACTATGCTCCCTTAGTCCTCGCCCCGATGACAGAAGTTATAAA  
AGTCAATATTTGAACAATGGCCCTCAGCGGATTGGTAGGAAGTACAA  
AAAAGTCCGATTTATGGCATAACAGATGAAACCTTTAAGACTCGTG  
AAGCTATTGAGCATGAATCAGGAATCTTGGGACCTTTACTTTATGGGG  
AAGTTGGAGACACACTGTTGATTATATTTAAGAATCAAGCAAGCAGA  
CCATATAACATCTACCCTCACGGAATCACTGATGTCCGTCCTTTGTAT  
TCAAGGAGATTACCAAAGGTGTAAACATTTGAAGGATTTTCCAAT  
TCTGCCAGGAGAAATATTCAAATATAAATGGACAGTGAAGTAGAAG  
ATGGGCCAACTAAATCAGATCCTCGGTGCCTGACCCGCTATTACTCTA  
GTTTCGTTAATATGGAGAGAGATCTAGCTTCAGGACTCATTGGCCCTC  
TCCTCATCTGCTACAAAGAATCTGTAGATCAAAGAGGAAACCAGATA  
ATGTCAGACAAGAGGAATGTCATCCTGTTTTCTGTATTTGATGAGAAC  
CGAAGCTGGTACCTCACAGAGAATATACAACGCTTTCTCCCAATCCA  
GCTGGAGTGCAGCTTGAGGATCCAGAGTTCCAAGCCTCCAACATCAT  
GCACAGCATCAATGGCTATGTTTTTGATAGTTTGCAGTTGTCAGTTTG  
TTTGCATGAGGTGGCATACTGGTACATTCTAAGCATTGGAGCACAGA  
CTGACTTCCTTTCTGTCTTCTCTCTGGATATACCTTCAAACACAAAAT

299/498

FIG. 72A-2

GGTCTATGAAGACACACTCACCTATTCCCATTCTCAGGAGAACTGT  
CTTCATGTGCGATGGAAAACCCAGGTCTATGGATTCTGGGGTGCCACA  
ACTCAGACTTTCGGAACAGAGGCATGACCGCCTTACTGAAGGTTTCT  
AGTTGTGACAAGAACACTGGTGATTATTACGAGGACAGTTATGAAGA  
TATTCAGCATACTTGCTGAGTAAAAACAATGCCATTGAACCAAGAA  
GCTTCTCCCAGAATTCAAGACACCGTAGCACTAGGCAAAAGCAATTT  
AATGCCACCACAATTCCAGAAAATGACATAGAGAAGACTGACCCTTG  
GTTTGCACACAGAACACCTATGCCTAAAATACAAAATGTCTCCTCTA  
GTGATTTGTTGATGCTCTTGCGACAGAGTCCTACTCCACATGGGCTAT  
CCTTATCTGATCTCCAAGAAGCGAAATATGAGACTTTTTCTGATGATC  
CATCACCTGGAGCAATAGACAGTAATAACAGCCTGTCTGAAATGACA  
CACTTCAGGCCACAGCTCCATCACAGTGGGGACATGGTATTTACCCC  
TGAGTCAGGCCTCCAATTAAGATTAAATGAGAACTGGGGACAACCTG  
CAGCAACAGAGTTGAAGAACTTGATTTCAAAGTTTCTAGTACATCA  
AATAATCTGATTTCAACAATTCATCAGACAATTTGGCAGCAGGTACT  
GATAATACAAGTTCCTTAGGACCCCCAAGTATGCCAGTTCATTATGAT  
AGTCAATTAGATACCACTCTATTTGGCAAAAAGTCATCTCCCCTTACT  
GAGTCTGGTGGACCTCTGAGCTTGAGTGAAGAAAATAATGATTCAAA  
GTTGTTAGAATCAGGTTTAATGAATAGCCAAGAAAGTTCATGGGGAA  
AAAATGTATCGTCAACAGAGAGTGGTAGGTTATTTAAAGGGAAAAGA  
GCTCATGGACCTGCTTTGTTGACTAAAGATAATGCCTTATTCAAAGTT  
AGCATCTCTTTGTTAAAGACAAACAAAACCTTCCAATAATTCAGCAACT  
AATAGAAAGACTCACATTGATGGCCCATCATTATTAATTGAGAATAG  
TCCATCAGTCTGGCAAAATATATTAGAAAGTGACACTGAGTTTAAAA  
AAGTGACACCTTTGATTCATGACAGAATGCTTATGGACAAAAATGCT  
ACAGCTTTGAGGCTAAATCATATGTCAAATAAAACTACTTCATCAAA  
AAACATGGAAATGGTCCAACAGAAAAAAGAGGGCCCCATTCCACCA  
GATGCACAAAATCCAGATATGTCGTTCTTTAAGATGCTATTCTTGCCA  
GAATCAGCAAGGTGGATACAAAGGACTCATGGAAAGAACTCTCTGAA  
CTCTGGGCAAGGCCCCAGTCCAAAGCAATTAGTATCCTTAGGACCAG  
AAAAATCTGTGGAAGGTCAGAATTTCTTGTCTGAGAAAAACAAAGTG  
GTAGTAGGAAAGGGTGAATTTACAAAGGACGTAGGACTCAAAGAGA  
TGGTTTTTCCAAGCAGCAGAAACCTATTTCTTACTAACTTGGATAATT  
TACATGAAAATAATACACACAATCAAGAAAAAAAATTCAGGAAGA  
AATAGAAAAGAAGGAAACATTAATCCAAGAGAATGTAGTTTTGCCTC  
AGATACATACAGTGACTGGCACTAAGAATTCATGAAGAACCTTTTC  
TACTGAGCACTAGGCAAAATGTAGAAGGTTTCATATGACGGGGCATA  
TGCTCCAGTACTTCAAGATTTTAGGTCATTAAATGATTCAACAAATAG  
AACAAAGAAACACACAGCTCATTCTCAAAAAAAGGGGAGGAAGAA  
AACTTGGAAGGCTTGGGAAATCAAACCAGCAAATTGTAGAGAAATAT  
GCATGCACCACAAGGAATATCTCCTAATAACAAGCCAGCAGAATTTTG  
TCACGCAACGTAGTAAGAGAGCTTTGAAACAATTCAGACTCCCCTA

FIG. 72A-3

300/498

GAAGAAACAGAACTTGAAAAAAGGATAATTGTGGATGACACCTCAAC  
CCAGTGGTCCAAAAACATGAAACATTTGACCCCGAGCACCTCACAC  
AGATAGACTACAATGAGAAGGAGAAAGGGGCCATTACTCAGTCTCCC  
TTATCAGATTGCCTTACGAGGAGTCATAGCATCCCTCAAGCAAATAGA  
TCTCCATTACCCATTGCAAAGGTATCATCATTTCCATCTATTAGACCTA  
TATATCTGACCAGGGTCCTATTCCAAGACAACTCTTCTCATCTTCCAG  
CAGCATCTTATAGAAAGAAAGATTCTGGGGTCCAAGAAAGCAGTCAT  
TTCTTACAAGGAGCCAAAAAAATAACCTTTCTTTAGCCATTCTAACC  
TTGGAGATGACTGGTGATCAAAGAGAGGTTGGCTCCCTGGGGACAAG  
TGCCACAAATTCAGTCACATACAAGAAAGTTGAGAACACTGTTCTCCC  
GAAACCAGACTTGCCCAAAACATCTGGCAAAGTTGAATTGCTTCCAA  
AAGTTCACATTTATCAGAAGGAGCTATTCCCTACGGAAACTAGCAATG  
GGTCTCCTGGCCATCTGGATCTCGTGGAAGGGAGCCTTCTTCAGGGAA  
CAGAGGGAGCGATTAAGTGGAATGAAGCAAACAGACCTGGAAAAGT  
TCCCTTTCTGAGAGTAGCAACAGAAAGCTCTGCAAAGACTCCCTCCAA  
GCTATTGGATCCTCTTGCTTGGGATAACCACTATGGTACTCAGATACC  
AAAAGAAGAGTGGAAATCCCAAGAGAAGTCACCAGAAAAAACAGCT  
TTAAGAAAAAAGGATACCATTTTGTCCCTGAACGCTTGTGAAAGCAAT  
CATGCAATAGCAGCAATAAATGAGGGACAAAATAAGCCCGAAATAG  
AAGTCACCTGGGCAAAGCAAGGTAGGACTGAAAGGCTGTGCTCTCAA  
AACCCACCAGTCTTGAAACGCCATCAACGGGAAATAACTCGTACTAC  
TCTTCAGTCAGATCAAGAGGAAATTGACTATGATGATACCATATCAGT  
TGAAATGAAGAAGGAAGATTTTGACATTTATGATGAGGATGAAAATC  
AGAGCCCCCGCAGCTTTCAAAAGAAAACACGACACTATTTTATTGCTG  
CAGTGGAGAGGCTCTGGGATTATGGGATGAGTAGCTCCCCACATGTT  
CTAAGAAACAGGGCTCAGAGTGGCAGTGTCCCTCAGTTCAAGAAAGT  
TGTTTTCCAGGAATTTACTGATGGCTCCTTTACTCAGCCCTTATACCGT  
GGAGAATAAATGAACATTTGGGACTCCTGGGGCCATATATAAGAGC  
AGAAGTTGAAGATAATATCATGGTAACTTTCAGAAATCAGGCCTCTC  
GTCCCTATTCCTTCTATTCTAGCCTTATTTCTTATGAGGAAGATCAGAG  
GCAAGGAGCAGAACCTAGAAAAAAGCTTTGTCAAGCCTAATGAAACCA  
AACTTACTTTTGAAAGGTGCAACATCATATGGCACCCACTAAAGAT  
GAGTTTGACTGCAAAGCCTGGGCTTATTTCTCTGATGTTGACCTGGAA  
AAAGATGTGCACTCAGGCCTGATTGGACCCCTTCTGGTCTGCCCACT  
AACACACTGAACCCTGCTCATGGGAGACAAGTGACAGTACAGGAATT  
TGCTCTGTTTTTACCATCTTTGATGAGACCAAAAGCTGGTACTTCACT  
GAAAATATGGAAAGAACTGCAGGGCTCCCTGCAATATCCAGATGGA  
AGATCCCACTTTTAAAGAGAATTATCGCTTCCATGCAATCAATGGCTA  
CATAATGGATACACTACCTGGCTTAGTAATGGCTCAGGATCAAAGGA  
TTCGATGGTATCTGCTCAGCATGGGCAGCAATGAAAACATCCATTCT  
ATTCATTTCAAGTGGACATGTGTTCACTGTACGAAAAAAAGAGGAGTA  
TAAATGGCACTGTACAATCTCTATCCAGGTGTTTTTGAGACAGTGGA

301/498

FIG. 72A-4

AATGTTACCATCCAAAGCTGGAATTTGGCGGGTGGAATGCCTTATTGG  
CGAGCATCTACATGCTGGGATGAGCACACTTTTTCTGGTGTACAGCAA  
TAAGTGTGAGACTCCCCTGGGAATGGCTTCTGGACACATTAGAGATTT  
TCAGATTACAGCTTCAGGACAATATGGACAGTGGGCCCCAAAGCTGG  
CCAGACTTCATTATTCCGGATCAATCAATGCCTGGAGCACCAAGGAG  
CCCTTTTCTTGGATCAAGGTGGATCTGTTGGCACCAATGATTATTCAC  
GGCATCAAGACCCAGGGTGCCCGTCAGAAGTTCTCCAGCCTCTACAT  
CTCTCAGTTTATCATCATGTATAGTCTTGATGGGAAGAAGTGGCAGA  
CTTATCGAGGAAATTCCACTGGAACCTTAATGGTCTTCTTTGGCAATG  
TGGATTTCATCTGGGATAAAACACAATATTTTAAACCCTCCAATTATTG  
CTCGATACATCCGTTTGCACCCAACTCATTATAGCATTTCGCAGCACTC  
TTCGCATGGAGTTGATGGGCTGTGATTTAAATAGTTGCAGCATGCCAT  
TGGGAATGGAGAGTAAAGCAATATCAGATGCACAGATTACTGCTTCA  
TCCTACTTTACCAATATGTTTGCCACCTGGTCTCCTTCAAAAGCTCGA  
CTTCACCTCCAAGGGAGGAGTAATGCCTGGAGACCTCAGGTGAATAA  
TCCAAAAGAGTGGCTGCAAGTGGACTTCCAGAAGACAATGAAAGTCA  
CAGGAGTAACTACTCAGGGAGTAAATCTCTGCTTACCAGCATGTAT  
GTGAAGGAGTTCCTCATCTCCAGCAGTCAAGATGGCCATCAGTGGAC  
TCTCTTTTTTCAGAATGGCAAAGTAAAGGTTTTTCAGGGAAATCAAGA  
CTCCTTCACACCTGTGGTGAACCTCTCTAGACCCACCGTTACTGACTCG  
CTACCTTCGAATTCACCCCCAGAGTTGGGTGCACCAGATTGCCCTGAG  
GATGGAGGTTCTGGGCTGCGAGGCACAGGACCTCTACTGAGGGTGGC  
CACTGCAGCACCTGCCACTGCCGTACCTCTCCCTCCTCAGCTCCAGG  
GCAGTGTCCCTCCCTGGCTTGCCTTCTACCTTTGTGCTAAATCCTAGC  
AGACACTGCCTTGAAGCCTCCTGAATTAATCATCAGTCCTGCATT  
TCTTTGGTGGGGGGCCAGGAGGGTGCATCCAATTTAACTTAACTCTTA  
CCTATTTTCTGCAGCTGCTCCCAGATTACTCCTTCCTTCCAATATAACT  
AGGCAAAAAGAAGTGAGGAGAAACCTGCATGAAAGCATTCTTCCCTG  
AAAAGTTAGGCCTCTCAGAGTCACCACTTCCTCTGTTGTAGAAAACT  
ATGTGATGAACTTTGAAAAAGATATTTATGATGTTAACATTTTCAGGT  
TAAGCCTCATACGTTTAAAATAAACTCTCAGTTGTTTATTATCCTGA  
TCAAGCATGGAACAAAGCATGTTTCAGGATCAGATCAATACAATCTT  
GGAGTCAAAAGGCAAATCATTTGGACAATCTGCAAAATGGAGAGAA  
TACAATAACTACTACAGTAAAGTCTGTTTCTGCTTCCTTACACATAGA  
TATAATTATGTTATTTAGTCATTATGAGGGGCACATTCTTATCTCCAA  
AACTAGCATTCTTAACTGAGAATTATAGATGGGGTTCAAGAATCCC  
TAAGTCCCCTGAAATTATATAAGGCATTCTGTATAAATGCAAATGTGC  
ATTTTTCTGACGAGTGTCCATAGATATAAAGCCATTTGGTCTTAATTCT  
GACCAATAAAAAAATAAGTCAGGAGGATGCAATTGTTGAAAGCTTTG  
AAATAAAATAACAATGTCTTCTTGAAATTTGTGATGGCCAAGAAAGA  
AAATGATGA

FIG. 72B-1

302/498

Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe Cys Phe Ser  
Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr Met Gln Ser  
Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro Arg Val Pro Lys Ser Phe  
Pro Phe Asn Thr Ser Val Val Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His  
Leu Phe Asn Ile Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile  
Gln Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro  
Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala Glu Tyr  
Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val Phe Pro Gly Gly  
Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn Gly Pro Met Ala Ser Asp Pro  
Leu Cys Leu Thr Tyr Ser Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn  
Ser Gly Leu Ile Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys  
Thr Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser  
Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser Ala Arg  
Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg Ser Leu Pro Gly Leu  
Ile Gly Cys His Arg Lys Ser Val Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro  
Glu Val His Ser Ile Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln  
Ala Ser Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp  
Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met Glu  
Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn  
Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met Asp Val  
Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys  
Lys His Pro Lys Thr Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr  
Ala Pro Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn  
Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr  
Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile Leu Gly Pro  
Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser  
Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg  
Arg Leu Pro Lys Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile  
Phe Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg  
Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala Ser  
Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp Gln Arg Gly Asn  
Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe Ser Val Phe Asp Glu Asn Arg  
Ser Trp Tyr Leu Thr Glu Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln  
Leu Glu Asp Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val  
Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu  
Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr Thr Phe Lys  
His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro Phe Ser Gly Glu Thr Val  
Phe Met Ser Met Glu Asn Pro Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe

## FIG. 72B-2 303/498

Arg Asn Arg Gly Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr  
Gly Asp Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn  
Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg Gln  
Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys Thr Asp Pro Trp  
Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn Val Ser Ser Ser Asp Leu Leu  
Met Leu Leu Arg Gln Ser Pro Thr Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu  
Ala Lys Tyr Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn  
Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val  
Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly Thr Thr  
Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser Thr Ser Asn Asn Leu  
Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala Gly Thr Asp Asn Thr Ser Ser Leu  
Gly Pro Pro Ser Met Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly  
Lys Lys Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn  
Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp Gly  
Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys Arg Ala His Gly  
Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys Val Ser Ile Ser Leu Leu  
Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala Thr Asn Arg Lys Thr His Ile Asp  
Gly Pro Ser Leu Leu Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser  
Asp Thr Glu Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp  
Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr Thr Ser Ser  
Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly Pro Ile Pro Pro Asp Ala  
Gln Asn Pro Asp Met Ser Phe Phe Lys Met Leu Phe Leu Pro Glu Ser Ala Arg  
Trp Ile Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro  
Lys Gln Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe Leu  
Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr Lys Asp Val Gly  
Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp  
Asn Leu His Glu Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile  
Glu Lys Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr  
Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr Arg Gln  
Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu Gln Asp Phe Arg  
Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys His Thr Ala His Phe Ser Lys  
Lys Gly Glu Glu Glu Asn Leu Glu Gly Leu Gly Asn Gln Thr Lys Gln Ile Val  
Glu Lys Tyr Ala Cys Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe  
Val Thr Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu  
Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser Lys Asn  
Met Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys Glu  
Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile  
Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser Ile  
Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser Ser His Leu Pro

## FIG. 72B-3 304/498

Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu  
Gln Gly Ala Lys Lys Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr  
Gly Asp Gln Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr  
Tyr Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr Ser  
Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys Asp Leu Phe Pro  
Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu Asp Leu Val Glu Gly Ser Leu  
Leu Gln Gly Thr Glu Gly Ala Ile Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys  
Val Pro Phe Leu Arg Val Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser Lys Leu  
Leu Asp Pro Leu Ala Trp Asp Asn His Tyr Gly Thr Gln Ile Pro Lys Glu Glu  
Trp Lys Ser Gln Glu Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr Ile  
Leu Ser Leu Asn Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly  
Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg  
Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg His Gln Arg Glu Ile Thr Arg  
Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu  
Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg  
Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp  
Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val  
Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro  
Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg  
Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro  
Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu  
Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val  
Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr  
Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu  
Leu Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln  
Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu  
Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr  
Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro  
Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly  
Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys  
Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr  
Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu  
His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr  
Pro Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln  
Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala  
Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile  
Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser  
Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly  
Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile

FIG. 72B-4

305/498

Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr  
His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn  
Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr  
Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala Arg Leu  
His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro Lys Glu  
Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln  
Gly Val Lys Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser  
Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe  
Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu  
Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg  
Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr



306/498

FIG. 73A

TCCACCTGTCCCCGCAGCGCCGGGCTCGCGCCCTCCTGCCGCAGCCACC  
GAGCCGCCGTCTAGCGCCCCGACCTCGCCACCATGAGAGCCCTGCTG  
GCGCGCCTGCTTCTCTGCGTCCTGGTCGTGAGCGACTCCAAAGGCAGC  
AATGAAC TTCATCAAGTTCCATCGAACTGTGACTGTCTAAATGGAGGA  
ACATGTGTGTCCAACAAGTACTTCTCCAACATTCACTGGTGCAACTGC  
CCAAAGAAATTCGGAGGGGCAGCACTGTGAAATAGATAAGTCAAAAAC  
CTGCTATGAGGGGAATGGTCACTTTTACCGAGGAAAGGCCAGCACTG  
ACACCATGGGCGCGCCCTGCCTGCCCTGGA ACTCTGCCACTGTCCTTC  
AGCAAACGTACCATGCCCACAGATCTGATGCTCTTCAGCTGGGCCTGG  
GGAAACATAATTACTGCAGGAACCCAGACAACCGGAGGCGACCCCTGG  
TGCTATGTGCAGGTGGGCCTAAAGCCGCTTGTCCAAGAGTGCATGGT  
GCATGACTGCGCAGATGGAAAAAAGCCCTCCTCTCCTCCAGAAGAAT  
TAAAATTTCAGTGTGGCCAAAAGACTCTGAGGCCCCGCTTTAAGATTA  
TTGGGGGAGAAATTCACCACCATCGAGAACCAGCCCTGGTTTTCGGGCC  
ATCTACAGGAGGCACCGGGGGGGCTCTGTACCTACGTGTGTGGAGG  
CAGCCTCATCAGCCCTTGCTGGGTGATCAGCGCCACACACTGCTTCAT  
TGATTACCCAAAGAAGGAGGACTACATCGTCTACCTGGGTTCGCTCAA  
GGCTTAACTCCAACACGCAAGGGGAGATGAAGTTTGAGGTGGAAAAC  
CTCATCCTACACAAGGACTACAGCGCTGACACGCTTGCTCACCACAAC  
GACATTGCCTTGCTGAAGATCCGTTCCAAGGAGGGCAGGTGTGCGCA  
GCCATCCCGGACTATACAGACCATCTGCCTGCCCTCGATGTATAACGA  
TCCCCAGTTTGGCACAAGCTGTGAGATCACTGGCTTTGGAAAAGAGA  
ATTCTACCGACTATCTCTATCCGGAGCAGCTGAAGATGACTGTTGTGA  
AGCTGATTTCCACCGGGAGTGTGAGCAGCCCCACTACTACGGCTCTG  
AAGTCACCACCAAAATGCTGTGTGCTGCTGACCCACAGTGGAAAACA  
GATTCCTGCCAGGGAGACTCAGGGGGACCCCTCGTCTGTTCCCTCCAA  
GGCCGCATGACTTTGACTGGAATTGTGAGCTGGGGCCGTGGATGTGC  
CCTGAAGGACAAGCCAGGCGTCTACACGAGAGTCTCACACTTCTTAC  
CCTGGATCCGCAAGTCACACCAAGGAAGAGAATGGCCTGGCCCTCTGA  
GGGTCCCCAGGGAGGAAACGGGCACCAACCGCTTTCTTGCTGGTTGTC  
ATTTTTGCAGTAGAGTCATCTCCATCAGCTGTAAGAAGAGACTGGGA  
AGAT

307/498

FIG. 73B

Met Arg Ala Leu Leu Ala Arg Leu Leu Leu Cys Val Leu Val Val Ser Asp Ser  
Lys Gly Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly  
Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro Lys  
Lys Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys Thr Cys Tyr Glu Gly Asn  
Gly His Phe Tyr Arg Gly Lys Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro  
Trp Asn Ser Ala Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp Ala Leu  
Gln Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Asn Arg Arg Arg  
Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu Val Gln Glu Cys Met Val His  
Asp Cys Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu Glu Leu Lys Phe Gln Cys  
Gly Gln Lys Thr Leu Arg Pro Arg Phe Lys Ile Ile Gly Gly Glu Phe Thr Thr Ile  
Glu Asn Gln Pro Trp Phe Ala Ala Ile Tyr Arg Arg His Arg Gly Gly Ser Val Thr  
Tyr Val Cys Gly Gly Ser Leu Ile Ser Pro Cys Trp Val Ile Ser Ala Thr His Cys  
Phe Ile Asp Tyr Pro Lys Lys Glu Asp Tyr Ile Val Tyr Leu Gly Arg Ser Arg Leu  
Asn Ser Asn Thr Gln Gly Glu Met Lys Phe Glu Val Glu Asn Leu Ile Leu His Lys  
Asp Tyr Ser Ala Asp Thr Leu Ala His His Asn Asp Ile Ala Leu Leu Lys Ile Arg  
Ser Lys Glu Gly Arg Cys Ala Gln Pro Ser Arg Thr Ile Gln Thr Ile Cys Leu Pro  
Ser Met Tyr Asn Asp Pro Gln Phe Gly Thr Ser Cys Glu Ile Thr Gly Phe Gly Lys  
Glu Asn Ser Thr Asp Tyr Leu Tyr Pro Glu Gln Leu Lys Met Thr Val Val Lys  
Leu Ile Ser His Arg Glu Cys Gln Gln Pro His Tyr Tyr Gly Ser Glu Val Thr Thr  
Lys Met Leu Cys Ala Ala Asp Pro Gln Trp Lys Thr Asp Ser Cys Gln Gly Asp  
Ser Gly Gly Pro Leu Val Cys Ser Leu Gln Gly Arg Met Thr Leu Thr Gly Ile Val  
Ser Trp Gly Arg Gly Cys Ala Leu Lys Asp Lys Pro Gly Val Tyr Thr Arg Val Ser  
His Phe Leu Pro Trp Ile Arg Ser His Thr Lys Glu Glu Asn Gly Leu Ala Leu

308/498

FIG.74A

TCCTGCACAGGCAGTGCCTTGAAGTGCTTCTTCAGAGACCTTTCTTCA  
TAGACTACTTTTTTTTCTTTAAGCAGCAAAAGGAGAAAATTGTCATCA  
AGGATATTCCAGATTCTTGACAGCATTCTCGTCATCTCTGAGGACATC  
ACCATCATCTCAGGATGAGGGGCATGAAGCTGCTGGGGGGCGCTGCTG  
GCACTGGCGGCCCTACTGCAGGGGGCCGTGTCCCTGAAGATCGCAGC  
CTTCAACATCCAGACATTTGGGGAGACCAAGATGTCCAATGCCACCCT  
CGTCAGCTACATTGTGCAGATCCTGAGCCGCTATGACATCGCCCTGGT  
CCAGGAGGTCAGAGACAGCCACCTGACTGCCGTGGGGAAGCTGCTGG  
ACAACCTCAATCAGGATGCACCAAGACACCTATCACTACGTGGTCAGT  
GAGCCACTGGGACGGAACAGCTATAAGGAGCGCTACCTGTTTCGTGTA  
CAGGCCTGACCAGGTGTCTGCGGTGGACAGCTACTACTACGATGATG  
GCTGCGAGCCCTGCGGGAACGACACCTTCAACCGAGAGCCAGCCATT  
GTCAGGTTCTTCTCCCGGTTACAGAGGTCAGGGAGTTTGCCATTGTT  
CCCCTGCATGCGGCCCCGGGGGACGCAGTAGCCGAGATCGACGCTCT  
CTATGACGTCTACCTGGATGTCCAAGAGAAATGGGGCTTGAGGACG  
TCATGTTGATGGGCGACTTCAATGCGGGCTGCAGCTATGTGAGACCCT  
CCCAGTGGTCATCCATCCGCCTGTGGACAAGCCCCACCTTCCAGTGGC  
TGATCCCCGACAGCGCTGACACCACAGCTACACCCACGCACTGTGCCT  
ATGACAGGATCGTGGTTGCAGGGATGCTGCTCCGAGGCGCCGTTGTT  
CCGACTCGGCTCTTCCCTTTAACTTCCAGGCTGCCTATGGCCTGAGTG  
ACCAACTGGCCCAAGCCATCAGTGACCACTATCCAGTGGAGGTGATG  
CTGAAGTGAGCAGCCCCTCCCCACACCAGTTGAACTGCAG

309/498

FIG. 74B

Met Arg Gly Met Lys Leu Leu Gly Ala Leu Leu Ala Leu Ala Ala Leu Leu Gln  
Gly Ala Val Ser Leu Lys Ile Ala Ala Phe Asn Ile Gln Thr Phe Gly Glu Thr Lys  
Met Ser Asn Ala Thr Leu Val Ser Tyr Ile Val Gln Ile Leu Ser Arg Tyr Asp Ile  
Ala Leu Val Gln Glu Val Arg Asp Ser His Leu Thr Ala Val Gly Lys Leu Leu  
Asp Asn Leu Asn Gln Asp Ala Pro Asp Thr Tyr His Tyr Val Val Ser Glu Pro  
Leu Gly Arg Asn Ser Tyr Lys Glu Arg Tyr Leu Phe Val Tyr Arg Pro Asp Gln  
Val Ser Ala Val Asp Ser Tyr Tyr Tyr Asp Asp Gly Cys Glu Pro Cys Gly Asn  
Asp Thr Phe Asn Arg Glu Pro Ala Ile Val Arg Phe Phe Ser Arg Phe Thr Glu Val  
Arg Glu Phe Ala Ile Val Pro Leu His Ala Ala Pro Gly Asp Ala Val Ala Glu Ile  
Asp Ala Leu Tyr Asp Val Tyr Leu Asp Val Gln Glu Lys Trp Gly Leu Glu Asp  
Val Met Leu Met Gly Asp Phe Asn Ala Gly Cys Ser Tyr Val Arg Pro Ser Gln  
Trp Ser Ser Ile Arg Leu Trp Thr Ser Pro Thr Phe Gln Trp Leu Ile Pro Asp Ser  
Ala Asp Thr Thr Ala Thr Pro Thr His Cys Ala Tyr Asp Arg Ile Val Val Ala Gly  
Met Leu Leu Arg Gly Ala Val Val Pro Asp Ser Ala Leu Pro Phe Asn Phe Gln  
Ala Ala Tyr Gly Leu Ser Asp Gln Leu Ala Gln Ala Ile Ser Asp His Tyr Pro Val  
Glu Val Met Leu Lys

310/498

FIG. 75A

GCTGCATCAGAAGAGGCCATCAAGCACATCACTGTCCTTCTGCCATGG  
CCCTGTGGATGCGCCTCCTGCCCCTGCTGGCGCTGCTGGCCCTCTGGG  
GACCTGACCCAGCCGCAGCCTTTGTGAACCAACACCTGTGCGGCTCAC  
ACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCT  
ACACACCCAAGACCCGCGGGAGGCAGAGGACCTGCAGGTGGGGCA  
GGTGGAGCTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGG  
CCCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTGGAACAATGCTGT  
ACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAGACG  
CAGCCCGCAGGCAGCCCCCACC CGCCTCCTGCACCGAGAGAGA  
TGGAATAAAGCCCTTGAACCAGC

FIG. 75B

Met Ala Leu Trp Met Arg Leu Leu Pro Leu Leu Ala Leu Leu Ala Leu Trp Gly  
Pro Asp Pro Ala Ala Ala Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val  
Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr  
Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro  
Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile  
Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn

311/498

FIG. 76A

ATGGGAGGTTGGTCTTCCAAACCTCGACAAGGCATGGGGACGAATCT  
TTCTGTTCCCAATCCTCTGGGATTCTTTCCCGATCACCAGTTGGACCCT  
GCGTTCGGAGCCAACTCAAACAATCCAGATTGGGACTTCAACCCCAA  
CAAGGATCACTGGCCAGAGGCAATCAAGGTAGGAGCGGGAGACTTC  
GGGCCAGGGTTCACCCACACACGGCGGTCTTTTGGGGTGGAGCCC  
TCAGGCTCAGGGCATATTGACAACAGTGCCAGCAGCGCCTCCTCCTG  
TTCCACCAATCGGCAGTCAGGAAGACAGCCTACTCCCATCTCTCCAC  
CTCTAAGAGACAGTCATCCTCAGGCCATGCAGTGGAAGTCCACAACA  
TTCCACCAAGCTCTGCTAGATCCCAGAGTGAGGGGCCTATATTTTCCT  
GCTGGTGGCTCCAGTTCCGGAACAGTAAACCCTGTTCCGACTACTGTC  
TCACCCATATCGTCAATCTTCTCGAGGACTGGGGACCCTGCACCGAAC  
ATGGAGAGCACAACATCAGGATTCCTAGGACCCCTGCTCGTGTTACA  
GGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCACAGAGTCT  
AGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCACCCACGTG  
TCCTGGCCAAAATTTCGAGTCCCCAACCTCCAATCACTCACCAACCTC  
TTGTCTCCAATTTGTCTGCTGCTATCGCTGGATGTGTCTGCGGCGTTTT  
ATCATATTCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTC  
TTCTGGACTACCAAGGTATGTTGCCCGTTTGTCTCTACTTCCAGGAA  
CATCAACTACCAGCACGGGACCATGCAAGACCTGCACGATTCTGCT  
CAAGGAACCTCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGAC  
GGAAACTGCACTTGTATTCCCATCCCATCATCCTGGGCTTTCGCAAGA  
TTCTATGAGGAGTGGGCCTCAGTCCGTTTCTCCTGGCTCAGTTTACTA  
GTGCCATTTGTTCAAGTGGTTCGCAGGGCTTCCCCCACTGTTTGGCTTT  
CAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAACATCT  
TGAGTCCCTTTTTACCTCTATTACCAATTTTCTTTTGTCTTTGGGTATAC  
ATTTGA

312/498

FIG. 76B

Met Gly Gly Trp Ser Ser Lys Pro Arg Gln Gly Met Gly Thr Asn Leu Ser Val Pro  
Asn Pro Leu Gly Phe Phe Pro Asp His Gln Leu Asp Pro Ala Phe Gly Ala Asn  
Ser Asn Asn Pro Asp Trp Asp Phe Asn Pro Asn Lys Asp His Trp Pro Glu Ala Ile  
Lys Val Gly Ala Gly Asp Phe Gly Pro Gly Phe Thr Pro Pro His Gly Gly Leu Leu  
Gly Trp Ser Pro Gln Ala Gln Gly Ile Leu Thr Thr Val Pro Ala Ala Pro Pro Pro  
Val Ser Thr Asn Arg Gln Ser Gly Arg Gln Pro Thr Pro Ile Ser Pro Pro Leu Arg  
Asp Ser His Pro Gln Ala Met Gln Trp Asn Ser Thr Thr Phe His Gln Ala Leu Leu  
Asp Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly Gly Ser Ser Ser Gly Thr Val  
Asn Pro Val Pro Thr Thr Val Ser Pro Ile Ser Ser Ile Phe Ser Arg Thr Gly Asp  
Pro Ala Pro Asn Met Glu Ser Thr Thr Ser Gly Phe Leu Gly Pro Leu Leu Val Leu  
Gln Ala Gly Phe Phe Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp Ser  
Trp Trp Thr Ser Leu Asn Phe Leu Gly Gly Ala Pro Thr Cys Pro Gly Gln Asn Ser  
Gln Ser Pro Thr Ser Asn His Ser Pro Thr Ser Cys Pro Pro Ile Cys Pro Gly Tyr  
Arg Trp Met Cys Leu Arg Arg Phe Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu  
Ile Phe Leu Leu Val Leu Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu  
Leu Pro Gly Thr Ser Thr Thr Ser Thr Gly Pro Cys Lys Thr Cys Thr Ile Pro Ala  
Gln Gly Thr Ser Met Phe Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly Asn  
Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Ala Arg Phe Leu Trp Glu Trp  
Ala Ser Val Arg Phe Ser Trp Leu Ser Leu Leu Val Pro Phe Val Gln Trp Phe Ala  
Gly Leu Ser Pro Thr Val Trp Leu Ser Val Ile Trp Met Met Trp Tyr Trp Gly Pro  
Ser Leu Tyr Asn Ile Leu Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu  
Trp Val Tyr Ile

313/498

FIG. 77A

CGAACCCTCAGGGTCCTGTGGACAGCTCACCTAGCTGCAATGGCTA  
CAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGCCTGC  
CCTGGCTTCAAGAGGGCAGTGCCTTCCCAACCATTCCTTATCCAGGC  
CTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTGGCCT  
TTGACACCTACCAGGAGTTTGAAGAAGCCTATATCCCAAAGGAACAG  
AAGTATTCATTCCCTGCAGAACCCCCAGACCTCCCTCTGTTTCTCAGAG  
TCTATTCCGACACCCTCCAACAGGGAGGAAACACAACAGAAATCCAA  
CCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGA  
GCCCCGTGCAGTTCCTCAGGAGTGTCTTCGCCAACAGCCTGGTGTACGG  
CGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAAG  
GCATCCAAACGCTGATGGGGAGGCTGGAAGATGGCAGCCCCCGGACT  
GGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACAACTCACA  
CAACGATGACGCACTACTCAAGAACTACGGGGCTGCTCTACTGCTTCAG  
GAAGGACATGGCAAGGTCGAGACATTCTGCGCATCGTGCAGTGCCG  
CTCTGTGGAGGGCAGCTGTGGCTTCTAGCTGCCCGGGTGCCATCCCTG  
TGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGT  
GCCACACAGCCTTGTCCTAATAAAATTAAGTTGCATC

FIG. 77B

Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu  
Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu Ser Arg Pro Phe Asp  
Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln  
Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro  
Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr  
Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp  
Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala  
Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu  
Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr  
Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly  
Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile  
Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe



314/498

FIG. 78A

ATGTATTCCAATGTGATAGGAACTGTAACCTCTGGAAAAAGGAAGGT  
TTATCTTTTGTCTTGCTGCTCATTGGCTTCTGGGACTGCGTGACCTGT  
CACGGGAGCCCTGTGGACATCTGCACAGCCAAGCCGCGGGACATTCC  
CATGAATCCCATGTGCATTTACCGCTCCCCGGAGAAGAAGGCAACTG  
AGGATGAGGGCTCAGAACAGAAGATCCCGGAGGCCACCAACCGGCG  
TGTCTGGGAACTGTCCAAGGCCAATTCCCGCTTTGCTACCACTTTCTA  
TCAGCACCTGGCAGATTCCAAGAATGACAATGATAACATTTTCCTGTC  
ACCCCTGAGTATCTCCACGGCTTTTGCTATGACCAAGCTGGGTGCCTG  
TAATGACACCCTCCAGCAACTGATGGAGGTATTTAAGTTTGACACCAT  
ATCTGAGAAAACATCTGATCAGATCCACTTCTTCTTTGCCAACTGAA  
CTGCCGACTCTATCGAAAAGCCAACAAATCCTCCAAGTTAGTATCAGC  
CAATCGCCTTTTTTGGAGACAAATCCCTTACCTTCAATGAGACCTACCA  
GGACATCAGTGAGTTGGTATATGGAGCCAAGCTCCAGCCCCTGGACT  
TCAAGGAAAATGCAGAGCAATCCAGAGCGGCCATCAACAAATGGGTG  
TCCAATAAGACCGAAGGCCGAATCACCGATGTCATTCCCTCGGAAGC  
CATCAATGAGCTCACTGTTCTGGTGCTGGTTAACACCATTACTTCAA  
GGGCTGTGGAAGTCAAAGTTCAGCCCTGAGAACACAAGGAAGGAAC  
TGTTCTACAAGGCTGATGGAGAGTCGTGTTTCAAGCATCTATGATGTACC  
AGGAAGGCAAGTTCCGTTATCGGCGCGTGGCTGAAGGCACCCAGGTG  
CTTGAGTTGCCCTTCAAAGGTGATGACATCACCATGGTCCTCATCTTG  
CCCAAGCCTGAGAAGAGCCTGGCCAAGGTGGAGAAGGAACTCACCCC  
AGAGGTGCTGCAGGAGTGGCTGGATGAATTGGAGGAGATGATGCTGG  
TGGTCCACATGCCCCGCTTCCGCATTGAGGACGGCTTCAGTTTGAAGG  
AGCAGCTGCAAGACATGGGCCTTGTCGATCTGTTTCAAGCCCTGAAAAG  
TCCAAACTCCCAGGTATTGTTGCAAGAAGGCCGAGATGACCTCTATGTC  
TCAGATGCATTCCATAAGGCATTCTTGAAGGTAAATGAAGAAGGCAG  
TGAAGCAGCTGCAAGTACCGCTGTTGTGATTGCTGGCCGTTTCGCTAAA  
CCCCAACAGGGTGACTTTCAAGGCCAACAGGCCTTTCCTGGTTTTTAT  
AAGAGAAGTTCCTCTGAACACTATTATCTTCATGGGCAGAGTAGCCA  
ACCCTTGTGTAAAGTAA

315/498

FIG. 78B

Met Tyr Ser Asn Val Ile Gly Thr Val Thr Ser Gly Lys Arg Lys Val Tyr Leu Leu  
Ser Leu Leu Leu Ile Gly Phe Trp Asp Cys Val Thr Cys His Gly Ser Pro Val Asp  
Ile Cys Thr Ala Lys Pro Arg Asp Ile Pro Met Asn Pro Met Cys Ile Tyr Arg Ser  
Pro Glu Lys Lys Ala Thr Glu Asp Glu Gly Ser Glu Gln Lys Ile Pro Glu Ala Thr  
Asn Arg Arg Val Trp Glu Leu Ser Lys Ala Asn Ser Arg Phe Ala Thr Thr Phe Tyr  
Gln His Leu Ala Asp Ser Lys Asn Asp Asn Asp Asn Ile Phe Leu Ser Pro Leu Ser  
Ile Ser Thr Ala Phe Ala Met Thr Lys Leu Gly Ala Cys Asn Asp Thr Leu Gln Gln  
Leu Met Glu Val Phe Lys Phe Asp Thr Ile Ser Glu Lys Thr Ser Asp Gln Ile His  
Phe Phe Phe Ala Lys Leu Asn Cys Arg Leu Tyr Arg Lys Ala Asn Lys Ser Ser  
Lys Leu Val Ser Ala Asn Arg Leu Phe Gly Asp Lys Ser Leu Thr Phe Asn Glu  
Thr Tyr Gln Asp Ile Ser Glu Leu Val Tyr Gly Ala Lys Leu Gln Pro Leu Asp Phe  
Lys Glu Asn Ala Glu Gln Ser Arg Ala Ala Ile Asn Lys Trp Val Ser Asn Lys Thr  
Glu Gly Arg Ile Thr Asp Val Ile Pro Ser Glu Ala Ile Asn Glu Leu Thr Val Leu  
Val Leu Val Asn Thr Ile Tyr Phe Lys Gly Leu Trp Lys Ser Lys Phe Ser Pro Glu  
Asn Thr Arg Lys Glu Leu Phe Tyr Lys Ala Asp Gly Glu Ser Cys Ser Ala Ser  
Met Met Tyr Gln Glu Gly Lys Phe Arg Tyr Arg Arg Val Ala Glu Gly Thr Gln  
Val Leu Glu Leu Pro Phe Lys Gly Asp Asp Ile Thr Met Val Leu Ile Leu Pro Lys  
Pro Glu Lys Ser Leu Ala Lys Val Glu Lys Glu Leu Thr Pro Glu Val Leu Gln Glu  
Trp Leu Asp Glu Leu Glu Glu Met Met Leu Val Val His Met Pro Arg Phe Arg  
Ile Glu Asp Gly Phe Ser Leu Lys Glu Gln Leu Gln Asp Met Gly Leu Val Asp  
Leu Phe Ser Pro Glu Lys Ser Lys Leu Pro Gly Ile Val Ala Glu Gly Arg Asp Asp  
Leu Tyr Val Ser Asp Ala Phe His Lys Ala Phe Leu Glu Val Asn Glu Glu Gly Ser  
Glu Ala Ala Ala Ser Thr Ala Val Val Ile Ala Gly Arg Ser Leu Asn Pro Asn Arg  
Val Thr Phe Lys Ala Asn Arg Pro Phe Leu Val Phe Ile Arg Glu Val Pro Leu Asn  
Thr Ile Ile Phe Met Gly Arg Val Ala Asn Pro Cys Val Lys

316/498

FIG. 79A

ATGGATTACTACAGAAAATATGCAGCTATCTTTCTGGTCACATTGTCG  
GTGTTTCTGCATGTTCTCCATTCCGCTCCTGATGTGCAGGATTGCCCAG  
AATGCACGCTACAGGAAAACCCATTCTTCTCCCAGCCGGGTGCCCA  
ATACTTCAGTGCATGGGCTGCTGCTTCTCTAGAGCATATCCCCTCCA  
CTAAGGTCCAAGAAGACGATGTTGGTCCAAAAGAACGTCACCTCAGA  
GTCCACTTGCTGTGTAGCTAAATCATATAACAGGGTCACAGTAATGGG  
GGGTTTCAAAGTGGAGAACCACACGGCGTGCCACTGCAGTACTTGTT  
ATTATCACAAATCTTAA

FIG. 79B

Met Asp Tyr Tyr Arg Lys Tyr Ala Ala Ile Phe Leu Val Thr Leu Ser Val Phe Leu  
His Val Leu His Ser Ala Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu  
Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe  
Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn  
Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met  
Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His  
Lys Ser

317/498

FIG. 79C

ATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGC  
GGGACATGGGCATCCAAGGAGCCGCTTCGGCCACGGTGCCGCCCCAT  
CAATGCCACCCTGGCTGTGGAGAAGGAGGGCTGCCCCGTGTGCATCA  
CCGTCAACACCACCATCTGTGCCGGCTACTGCCCCACCATGACCCGCG  
TGCTGCAGGGGGTCCCTGCCGGCCCTGCCTCAGGTGGTGTGCAACTACC  
GCGATGTGCGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCGCGCGGCG  
TGAACCCCGTGGTCTCCTACGCCGTGGCTCTCAGCTGTCAATGTGCAC  
TCTGCCGCCGCAGCACCCTGACTGCGGGGGTCCCAAGGACCACCCC  
TTGACCTGTGATGACCCCCGCTTCCAGGACTCCTCTTCCTCAAAGGCC  
CCTCCCCCAGCCTTCCAAAGCCATCCCAGTCCCGGGGCCCTCGGAC  
ACCCCGATCCTCC CACAATAA

FIG. 79D

Met Glu Met Phe Gln Gly Leu Leu Leu Leu Leu Leu Ser Met Gly Gly Thr  
Trp Ala Ser Lys Glu Pro Leu Arg Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala  
Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn Thr Thr Ile Cys Ala Gly  
Tyr Cys Pro Thr Met Thr Arg Val Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val  
Val Cys Asn Tyr Arg Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg  
Gly Val Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys  
Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu Thr Cys Asp  
Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser  
Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln

318/498

FIG. 80A

ATGCGTCCCCTGCGCCCCCGCGCCGCGCTGCTGGCGCTCCTGGCCTCG  
CTCCTGGCCGCGCCCCCGGTGGCCCCGGCCGAGGCCCGCACCTGGT  
GCAGGTGGACGCGGCCCGCGCGCTGTGGCCCCTGCGGGCGCTTCTGGA  
GGAGCACAGGCTTCTGCCCCCGCTGCCACACAGCCAGGCTGACCAG  
TACGTCCTCAGCTGGGACCAGCAGCTCAACCTCGCCTATGTGGGCGCC  
GTCCCTCACCGCGGCATCAAGCAGGTCCGGACCCACTGGCTGCTGGA  
GCTTGTCAACCACCAGGGGGTCCACTGGACGGGGCCTGAGCTACAAC  
TCACCCACCTGGACGGGTACTTGGACCTTCTCAGGGAGAACCAGCTCC  
TCCCAGGGTTTGAGCTGATGGGCAGCGCCTCGGGCCACTTCACTGACT  
TTGAGGACAAGCAGCAGGTGTTTGAGTGGAAGGACTTGGTCTCCAGC  
CTGGCCAGGAGATACATCGGTAGGTACGGACTGGCGCATGTTTCCAA  
GTGGAACCTTCGAGACGTGGAATGAGCCAGACCACCACGACTTTGACA  
ACGTCTCCATGACCATGCAAGGCTTCCTGAACTACTACGATGCCTGCT  
CGGAGGGTCTGCGCGCCGCCAGCCCCGCCCTGCGGCTGGGAGGCCCC  
GGCGACTCCTTCCACACCCCAACGCGATCCCCGCTGAGCTGGGGCCTC  
CTGCGCCACTGCCACGACGGTACCAACTTCTTCACTGGGGAGGCGGG  
CGTGCGGCTGGACTACATCTCCCTCCACAGGAAGGGTGCGCGCAGCT  
CCATCTCCATCCTGGAGCAGGAGAAGGTCGTCGCGCAGCAGATCCGG  
CAGCTCTTCCCCAAGTTCGCGGACACCCCCATTTACAACGACGAGGCG  
GACCCGCTGGTGGGCTGGTCCCTGCCACAGCCGTGGAGGGCGGACGT  
GACCTACGCGGCCATGGTGGTGAAGGTCATCGCGCAGCATCAGAACC  
TGCTACTGGCCAAACACCACCTCCGCCTTCCCCTACGCGCTCCTGAGCA  
ACGACAATGCCTTCCTGAGCTACCACCCGCACCCCTTCGCGCAGCGCA  
CGCTCACCGCGCGCTTCCAGGTCAACAACACCCGCCCGCCGCACGTG  
CAGCTGTTGCGCAAGCCGGTGCTCACGGCCATGGGGCTGCTGGCGCT  
GCTGGATGAGGAGCAGCTCTGGGCCGAAGTGTCGCAGGCCGGGACCG  
TCCTGGACAGCAACCACACGGTGGGCGTCTGGCCAGCGCCACCGC  
CCCCAGGGCCCCGGCCGACGCCTGGCGCGCCGCGGTGCTGATCTACGC  
GAGCGACGACACCCGCGCCACCCCAACCGCAGCGTCGCGGTGACCC  
TGCGGCTGCGCGGGGTGCCCCCGGCCCGGGCCTGGTCTACGTCACG  
CGCTACCTGGACAACGGGCTCTGCAGCCCCGACGGCGAGTGGCGGCG  
CCTGGGCCCGGCCGTCTTCCCCACGGCAGAGCAGTTCCGGCGCATGC  
GCGCGGCTGAGGACCCGGTGGCCGCGGCGCCCCGCCCTTACCCGCC  
GGCGGCCGCTGACCCTGCGCCCCGCGCTGCGGCTGCCGTCGCTTTTG  
CTGGTGCACGTGTGTGCGCGCCCCGAGAAGCCGCCCGGGCAGGTAC  
GCGGCTCCGCGCCCTGCCCTGACCCAAGGGCAGCTGGTTCTGGTCTG  
GTCGGATGAACACGTGGGCTCCAAGTGCCTGTGGACATACGAGATCC  
AGTTCTCTCAGGACGGTAAGGCGTACACCCCGGTCAGCAGGAAGCCA  
TCGACCTTCAACCTCTTTGTGTTACGCCAGACACAGGTGCTGTCTCT  
GGCTCCTACCGAGTTCGAGCCCTGGACTACTGGGCCCGACCAAGGCC  
CTTCTCGGACCCTGTGCCGTACCTGGAGGTCCCTGTGCCAAGAGGGCC  
CCCATCCCCGGGCAATCCAT GA

319/498

FIG. 80B

Met Arg Pro Leu Arg Pro Arg Ala Ala Leu Leu Ala Leu Leu Ala Ser Leu Leu  
Ala Ala Pro Pro Val Ala Pro Ala Glu Ala Pro His Leu Val Gln Val Asp Ala Ala  
Arg Ala Leu Trp Pro Leu Arg Arg Phe Trp Arg Ser Thr Gly Phe Cys Pro Pro Leu  
Pro His Ser Gln Ala Asp Gln Tyr Val Leu Ser Trp Asp Gln Gln Leu Asn Leu Ala  
Tyr Val Gly Ala Val Pro His Arg Gly Ile Lys Gln Val Arg Thr His Trp Leu Leu  
Glu Leu Val Thr Thr Arg Gly Ser Thr Gly Arg Gly Leu Ser Tyr Asn Phe Thr His  
Leu Asp Gly Tyr Leu Asp Leu Leu Arg Glu Asn Gln Leu Leu Pro Gly Phe Glu  
Leu Met Gly Ser Ala Ser Gly His Phe Thr Asp Phe Glu Asp Lys Gln Gln Val  
Phe Glu Trp Lys Asp Leu Val Ser Ser Leu Ala Arg Arg Tyr Ile Gly Arg Tyr Gly  
Leu Ala His Val Ser Lys Trp Asn Phe Glu Thr Trp Asn Glu Pro Asp His His Asp  
Phe Asp Asn Val Ser Met Thr Met Gln Gly Phe Leu Asn Tyr Tyr Asp Ala Cys  
Ser Glu Gly Leu Arg Ala Ala Ser Pro Ala Leu Arg Leu Gly Gly Pro Gly Asp Ser  
Phe His Thr Pro Pro Arg Ser Pro Leu Ser Trp Gly Leu Leu Arg His Cys His Asp  
Gly Thr Asn Phe Phe Thr Gly Glu Ala Gly Val Arg Leu Asp Tyr Ile Ser Leu His  
Arg Lys Gly Ala Arg Ser Ser Ile Ser Ile Leu Glu Gln Glu Lys Val Val Ala Gln  
Gln Ile Arg Gln Leu Phe Pro Lys Phe Ala Asp Thr Pro Ile Tyr Asn Asp Glu Ala  
Asp Pro Leu Val Gly Trp Ser Leu Pro Gln Pro Trp Arg Ala Asp Val Thr Tyr Ala  
Ala Met Val Val Lys Val Ile Ala Gln His Gln Asn Leu Leu Leu Ala Asn Thr Thr  
Ser Ala Phe Pro Tyr Ala Leu Leu Ser Asn Asp Asn Ala Phe Leu Ser Tyr His Pro  
His Pro Phe Ala Gln Arg Thr Leu Thr Ala Arg Phe Gln Val Asn Asn Thr Arg  
Pro Pro His Val Gln Leu Leu Arg Lys Pro Val Leu Thr Ala Met Gly Leu Leu Ala  
Leu Leu Asp Glu Glu Gln Leu Trp Ala Glu Val Ser Gln Ala Gly Thr Val Leu  
Asp Ser Asn His Thr Val Gly Val Leu Ala Ser Ala His Arg Pro Gln Gly Pro Ala  
Asp Ala Trp Arg Ala Ala Val Leu Ile Tyr Ala Ser Asp Asp Thr Arg Ala His Pro  
Asn Arg Ser Val Ala Val Thr Leu Arg Leu Arg Gly Val Pro Pro Gly Pro Gly Leu  
Val Tyr Val Thr Arg Tyr Leu Asp Asn Gly Leu Cys Ser Pro Asp Gly Glu Trp  
Arg Arg Leu Gly Arg Pro Val Phe Pro Thr Ala Glu Gln Phe Arg Arg Met Arg  
Ala Ala Glu Asp Pro Val Ala Ala Ala Pro Arg Pro Leu Pro Ala Gly Gly Arg Leu  
Thr Leu Arg Pro Ala Leu Arg Leu Pro Ser Leu Leu Leu Val His Val Cys Ala  
Arg Pro Glu Lys Pro Pro Gly Gln Val Thr Arg Leu Arg Ala Leu Pro Leu Thr Gln  
Gly Gln Leu Val Leu Val Trp Ser Asp Glu His Val Gly Ser Lys Cys Leu Trp Thr  
Tyr Glu Ile Gln Phe Ser Gln Asp Gly Lys Ala Tyr Thr Pro Val Ser Arg Lys Pro  
Ser Thr Phe Asn Leu Phe Val Phe Ser Pro Asp Thr Gly Ala Val Ser Gly Ser Tyr  
Arg Val Arg Ala Leu Asp Tyr Trp Ala Arg Pro Gly Pro Phe Ser Asp Pro Val Pro  
Tyr Leu Glu Val Pro Val Pro Arg Gly Pro Pro Ser Pro Gly Asn Pro

320/498

FIG. 81A

ATGCAGCTGAGGAACCCAGAACTACATCTGGGCTGCGCGCTTGCGCT  
TCGCTTCCTGGCCCTCGTTTCCTGGGACATCCCTGGGGCTAGAGCACT  
GGACAATGGATTGGCAAGGACGCCTACCATGGGCTGGCTGCACTGGG  
AGCGCTTCATGTGCAACCTTGACTGCCAGGAAGAGCCAGATTCCTGC  
ATCAGTGAGAAGCTCTTCATGGAGATGGCAGAGCTCATGGTCTCAGA  
AGGCTGGAAGGATGCAGGTTATGAGTACCTCTGCATTGATGACTGTTG  
GATGGCTCCCCAAAGAGATTCAGAAGGCAGACTTCAGGCAGACCCTC  
AGCGCTTTCCTCATGGGATTCGCCAGCTAGCTAATTATGTTACAGCA  
AAGGACTGAAGCTAGGGATTTATGCAGATGTTGGAAATAAAACCTGC  
GCAGGCTTCCCTGGGAGTTTTGGATACTACGACATTGATGCCAGACC  
TTTGCTGACTGGGGAGTAGATCTGCTAAAATTTGATGGTTGTTACTGT  
GACAGTTTGGAAAATTTGGCAGATGGTTATAAGCACATGTCCTTGGCC  
CTGAATAGGACTGGCAGAAGCATTGTGTACTCCTGTGAGTGGCCTCTT  
TATATGTGGCCCTTTCAAAAGCCCAATTATACAGAAATCCGACAGTAC  
TGCAATCACTGGCGAAATTTTGCTGACATTGATGATTCCTGGAAAAGT  
ATAAAGAGTATCTTGGACTGGACATCTTTTAACCAGGAGAGAATTGTT  
GATGTTGCTGGACCAGGGGGTTGGAATGACCCAGATATGTTAGTGAT  
TGGCAACTTTGGCCTCAGCTGGAATCAGCAAGTAACTCAGATGGCCCT  
CTGGGCTATCATGGCTGCTCCTTTATTCATGTCTAATGACCTCCGACA  
CATCAGCCCTCAAGCCAAAGCTCTCCTTCAGGATAAGGACGTAATTGC  
CATCAATCAGGACCCCTTGGGCAAGCAAGGGTACCAGCTTAGACAGG  
GAGACAACTTTGAAGTGTGGGAACGACCTCTCTCAGGCTTAGCCTGG  
GCTGTAGCTATGATAAACCGGCAGGAGATTGGTGGACCTCGCTCTTAT  
ACCATCGCAGTTGCTTCCCTGGGTAAAGGAGTGGCCTGTAATCCTGCC  
TGCTTCATCACACAGCTCCTCCCTGTGAAAAGGAAGCTAGGGTTCTAT  
GAATGGACTTCAAGGTTAAGAAGTCACATAAATCCCACAGGCACTGT  
TTTGCTTCAGCTAGAAAATACAATGCAGATGTCATTAAAAGACTTACT  
TTAA

321/498

FIG. 81B

Met Gln Leu Arg Asn Pro Glu Leu His Leu Gly Cys Ala Leu Ala Leu Arg Phe  
Leu Ala Leu Val Ser Trp Asp Ile Pro Gly Ala Arg Ala Leu Asp Asn Gly Leu Ala  
Arg Thr Pro Thr Met Gly Trp Leu His Trp Glu Arg Phe Met Cys Asn Leu Asp  
Cys Gln Glu Glu Pro Asp Ser Cys Ile Ser Glu Lys Leu Phe Met Glu Met Ala Glu  
Leu Met Val Ser Glu Gly Trp Lys Asp Ala Gly Tyr Glu Tyr Leu Cys Ile Asp Asp  
Cys Trp Met Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln  
Arg Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys Gly Leu Lys  
Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala Gly Phe Pro Gly Ser Phe  
Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe Ala Asp Trp Gly Val Asp Leu Leu Lys  
Phe Asp Gly Cys Tyr Cys Asp Ser Leu Glu Asn Leu Ala Asp Gly Tyr Lys His  
Met Ser Leu Ala Leu Asn Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro  
Leu Tyr Met Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys Asn  
His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile Lys Ser Ile Leu  
Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp Val Ala Gly Pro Gly Gly Trp  
Asn Asp Pro Asp Met Leu Val Ile Gly Asn Phe Gly Leu Ser Trp Asn Gln Gln  
Val Thr Gln Met Ala Leu Trp Ala Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp  
Leu Arg His Ile Ser Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala  
Ile Asn Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp Asn  
Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val Ala Met Ile Asn  
Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile Ala Val Ala Ser Leu Gly Lys  
Gly Val Ala Cys Asn Pro Ala Cys Phe Ile Thr Gln Leu Leu Pro Val Lys Arg Lys  
Leu Gly Phe Tyr Glu Trp Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr  
Val Leu Leu Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu



322/498

FIG. 82A

ATGGCGCCCGTCGCCGTCTGGGCGCGCTGGCCGTCGGACTGGAGCT  
CTGGGCTGCGGCGCACGCCTTGCCCGCCAGGTGGCATTACACCCTA  
CGCCCCGGAGCCCGGGAGCACATGCCGGCTCAGAGAATACTATGACC  
AGACAGCTCAGATGTGCTGCAGCAAATGCTCGCCGGGCCAACATGCA  
AAAGTCTTCTGTACCAAGACCTCGGACACCGTGTGTGACTCCTGTGAG  
GACAGCACATACACCCAGCTCTGGAAGTGGGTTCGCGAGTGCTTGAG  
CTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAACTCAAGCCTGCAC  
TCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCG  
CGCTGAGCAAGCAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCGCAAG  
TGCCGCCCGGGCTTCGGCGTGGCCAGACCAGGAAGTGAACATCAGA  
CGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCCAACAGACTTC  
ATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCAT  
CCCTGGGAATGCAAGCATGGATGCAGTCTGCACGTCCACGTCCCCCA  
CCCGGAGTATGGCCCCAGGGGCACTACACTTACCCAGCCAGTGTCC  
ACACGATCCCAACACACGCAGCCAAGTCCAGAAGCCAGCACTGCTCC  
AAGCACCTCCTTCCTGCTCCCAATGGGCCCCAGCCCCCAGCTGAAGG  
GAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGAC  
AGCCTTGGGTCTACTAATAATAGGAGTGGTGAAGTGTGTCATCATGAC  
CCAGGTGAAAAAGAAGCCCTTGTGCCTGCAGAGAGAAGCCAAGGTGC  
CTCACTTGCCTGCCGATAAGGCCCGGGGTACACAGGGCCCCGAGCAG  
CAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCCCTGGA  
GAGCTCGGCCAGTGCGTTGGACAGAAGGGCGCCCACTCGGAACCAGC  
CACAGGCACCAGGCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGC  
CAGCACCGGGAGCTCAGATTCTTCCCCTGGTGGCCATGGGACCCAGG  
TCAATGTCACCTGCATCGTGAACGTCTGTAGCAGCTCTGACCACAGCT  
CACAGTGCTCCTCCCAAGCCAGCTCCACAATGGGAGACACAGATTCC  
AGCCCCTCGGAGTCCCCGAAGGACGAGCAGGTCCCCTTCTCCAAGGA  
GGAATGTGCCTTTCGGTCACAGCTGGAGACGCCAGAGACCCCTGCTGG  
GGAGCACCGAAGAGAAGCCCCTGCCCTTGGAGTGCCTGATGCTGGG  
ATGAAGCCCAGTTAACCAGGCCGGTGTGGGCTGTGTGCGTAGCCAAGG  
TGGGCTGAGCCCTGGCAGGATGACCCTGCGAAGGGGCCCTGGTCCTT  
CCAGGC

323/498

FIG. 82B

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu Trp Ala Ala  
Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr Ala Pro Glu Pro Gly Ser  
Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln Thr Ala Gln Met Cys Cys Ser Lys  
Cys Ser Pro Gly Gln His Ala Lys Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys  
Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys  
Leu Ser Cys Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg  
Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln  
Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val  
Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro Gly Thr  
Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val  
Val Ala Ile Pro Gly Asn Ala Ser Met Asp Ala Val Cys Thr Ser Thr Ser Pro Thr  
Arg Ser Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln  
His Thr Gln Pro Thr Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro  
Met Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Phe Ala Leu Pro Val Gly  
Leu Ile Val Gly Val Thr Ala Leu Gly Leu Leu Ile Ile Gly Val Val Asn Cys Val  
Ile Met Thr Gln Val Lys Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val Pro  
His Leu Pro Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu Gln Gln His Leu Leu  
Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg  
Arg Ala Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly  
Glu Ala Arg Ala Ser Thr Gly Ser Ser Asp Ser Ser Pro Gly Gly His Gly Thr Gln  
Val Asn Val Thr Cys Ile Val Asn Val Cys Ser Ser Ser Asp His Ser Ser Gln Cys  
Ser Ser Gln Ala Ser Ser Thr Met Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro  
Lys Asp Glu Gln Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu  
Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro Leu Gly Val Pro  
Asp Ala Gly Met Lys Pro Ser

324/498

FIG. 83A

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val  
Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala Val Ala Trp Tyr Gln Gln  
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly  
Val Pro Ser Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser  
Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro  
Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys

FIG. 83B

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg  
Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr Tyr Ile His Trp Val Arg  
Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr  
Thr Arg Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys  
Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr  
Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln  
Gly Thr Leu Val Thr Val Ser Ser

325/498

FIG. 84A

Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln Thr Leu Thr  
Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser Gly Met Ser Val Gly Trp  
Ile Arg Gln Pro Ser Gly Lys Ala Leu Glu Trp Leu Ala Asp Ile Trp Trp Asp Asp  
Lys Lys Asp Tyr Asn Pro Ser Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser  
Lys Asn Gln Val Val Leu Lys Val Thr Asn Met Asp Pro Ala Asp Thr Ala Thr  
Tyr Tyr Cys Ala Arg Ser Met Ile Thr Asn Trp Tyr Phe Asp Val Trp Gly Ala Gly  
Thr Thr Val Thr Val Ser Ser

FIG. 84B

Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Val Gly Asp Arg Val  
Thr Ile Thr Cys Lys Cys Gln Leu Ser Val Gly Tyr Met His Trp Tyr Gln Gln Lys  
Pro Gly Lys Ala Pro Lys Leu Trp Ile Tyr Asp Thr Ser Lys Leu Ala Ser Gly Val  
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser  
Leu Gln Pro Asp Asp Phe Ala Thr Tyr Tyr Cys Phe Gln Gly Ser Gly Tyr Pro Phe  
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys

326/498

FIG. 85A

GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGA  
GAAAGAGTCAGTTTCTCCTGCAGGGCCAGTCAGTTCGTTGGCTCAAGC  
ATCCACTGGTATCAGCAAAGAACAAATGGTTCTCCAAGGCTTCTCATA  
AAGTATGCTTCTGAGTCTATGTCTGGGATCCCTTCCAGGTTTAGTGGC  
AGTGGATCAGGGACAGATTTTACTCTTAGCATCAACACTGTGGAGTCT  
GAAGATATTGCAGATTATTACTGTCAACAAAGTCATAGCTGGCCATTC  
ACGTTTCGGCTCGGGGACAAATTTGGAAGTAAAAGAAGTGAAGCTTGA  
GGAGTCTGGAGGAGGCTTGGTGCAACCTGGAGGATCCATGAAACTCT  
CCTGTGTTGCCTCTGGATTCAATTTTCAGTAACCACTGGATGAACTGGG  
TCCGCCAGTCTCCAGAGAAGGGGCTTGAGTGGGTTGCTGAAATTAGA  
TCAAAATCTATTAATTCTGCAACACATTATGCGGAGTCTGTGAAAGGG  
AGGTTCAACCATCTCAAGAGATGATTCCAAAAGTGCTGTCTACCTGCAA  
ATGACCGACTTAAGAACTGAAGACACTGGCGTTTATTACTGTTCCAGG  
AATTACTACGGTAGTACCTACGACTACTGGGGCCAAGGCACCACTCTC  
ACAGTCTCC

FIG. 85B

Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser Val Ser Pro Gly Glu Arg Val  
Ser Phe Ser Cys Arg Ala Ser Gln Phe Val Gly Ser Ser Ile His Trp Tyr Gln Gln  
Arg Thr Asn Gly Ser Pro Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Met Ser Gly  
Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn  
Thr Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser His Ser Trp Pro  
Phe Thr Phe Gly Ser Gly Thr Asn Leu Glu Val Lys Glu Val Lys Leu Glu Glu Ser  
Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Met Lys Leu Ser Cys Val Ala Ser Gly  
Phe Ile Phe Ser Asn His Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu  
Glu Trp Val Ala Glu Ile Arg Ser Lys Ser Ile Asn Ser Ala Thr His Tyr Ala Glu  
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ala Val Tyr  
Leu Gln Met Thr Asp Leu Arg Thr Glu Asp Thr Gly Val Tyr Tyr Cys Ser Arg  
Asn Tyr Tyr Gly Ser Thr Tyr Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser

327/498

FIG. 86A

ATGGAGACAGACACACTCCTGTTATGGGTGCTGCTGCTCTGGGTTCCA  
GGTTCCACTGGTGACGTCAGGCGAGGGCCCCGGAGCCTGCGGGGCAG  
GGACGCGCCAGCCCCACGCCCTGCGTCCCGGCCGAGTGCTTCGACC  
TGCTGGTCCGCCACTGCGTGCCCTGCGGGCTCCTGCGCACGCCGCGGC  
CGAAACCGGCCGGGGCCAGCAGCCCTGCGCCCAGGACGGCGCTGCAG  
CCGCAGGAGTCGGTGGGCGCGGGGGGCCGGCGAGGCGGCGGTTCGACA  
AAACTCACACATGCCACCCGTGCCAGCACCTGAACTCCTGGGGGGA  
CCGTCAGTCTTCCTCTTCCCCCAAACCCAAGGACACCCTCATGATC  
TCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGA  
AGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGAGGTGC  
ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTA  
CCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGG  
CAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCA  
TCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAG  
GTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGT  
CAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGT  
GGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACACTACAAGACCACG  
CCTCCCGTGTTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTC  
ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTC  
CGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCT  
CCCTGTCTCCCGGGAAATGA

328/498

FIG. 86B

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro Gly Ser  
Thr Gly Asp Val Arg Arg Gly Pro Arg Ser Leu Arg Gly Arg Asp Ala Pro Ala  
Pro Thr Pro Cys Val Pro Ala Glu Cys Phe Asp Leu Leu Val Arg His Cys Val Ala  
Cys Gly Leu Leu Arg Thr Pro Arg Pro Lys Pro Ala Gly Ala Ser Ser Pro Ala Pro  
Arg Thr Ala Leu Gln Pro Gln Glu Ser Val Gly Ala Gly Ala Gly Glu Ala Ala Val  
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser  
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu  
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp  
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr  
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn  
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys  
Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro  
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly  
Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn  
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys  
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met  
His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys

329/498

FIG. 87

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly Asp Arg Val  
Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln  
Lys Pro Asp Gly Ile Val Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly  
Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser  
Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro  
Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys

FIG. 88

Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Gly Pro Gly Thr Ser Val Arg  
Val Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Tyr Leu Ile Glu Trp Val Lys  
Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly Val Ile Tyr Pro Gly Ser Gly Gly  
Thr Asn Tyr Asn Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser  
Thr Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Asp Asp Ser Ala Val Tyr Phe  
Cys Ala Arg Arg Asp Gly Asn Tyr Gly Trp Phe Ala Tyr Trp Gly Arg Gly Thr  
Leu Val Thr Val Ser Ala

FIG. 89

Asp Ile Gln Met Thr Gln Thr Pro Ser Thr Leu Ser Ala Ser Val Gly Asp Arg Val  
Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln  
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly  
Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser  
Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu  
Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Val Lys

FIG. 90

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser Val Lys  
Val Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Tyr Leu Ile Glu Trp Val Arg  
Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile Gly Val Ile Tyr Pro Gly Ser Gly Gly  
Thr Asn Tyr Asn Glu Lys Phe Lys Gly Arg Val Thr Leu Thr Val Asp Glu Ser  
Thr Asn Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr  
Phe Cys Ala Arg Arg Asp Gly Asn Tyr Gly Trp Phe Ala Tyr Trp Gly Gln Gly  
Thr Leu Val Thr Val Ser Ser



330/498

FIG. 91

Asp Ile Gln Met Thr Gln Thr Pro Ser Thr Leu Ser Ala Ser Val Gly Asp Arg Val  
 Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln  
 Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly  
 Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser  
 Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu  
 Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Val Lys Arg Thr Val Ala Ala Pro  
 Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val  
 Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val  
 Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys  
 Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys  
 His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys  
 Ser Phe Asn Arg Gly Glu Cys

FIG. 92

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser Val Lys  
 Val Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Tyr Leu Ile Glu Trp Val Arg  
 Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile Gly Val Ile Tyr Pro Gly Ser Gly Gly  
 Thr Asn Tyr Asn Glu Lys Phe Lys Gly Arg Val Thr Leu Thr Val Asp Glu Ser  
 Thr Asn Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr  
 Phe Cys Ala Arg Arg Asp Gly Asn Tyr Gly Trp Phe Ala Tyr Trp Gly Gln Gly  
 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala  
 Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp  
 Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val  
 His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val  
 Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys  
 Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His  
 Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe  
 Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val  
 Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly  
 Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr  
 Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu  
 Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser  
 Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp  
 Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser  
 Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr  
 Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp  
 Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu  
 His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly

331/498

FIG. 93A

ATGGATTTTCAGGTGCAGATTATCAGCTTCCTGCTAATCAGTGCTTCA  
GTCATAATGTCCAGAGGGCAAATTGTTCTCTCCCAGTCTCCAGCAATC  
CTGTCTGCATCTCCAGGGGAGAAGGTCACAATGACTTGCAGGGCCAG  
CTCAAGTGTAAGTTACATCCACTGGTTCCAGCAGAAGCCAGGATCCTC  
CCCCAAACCCTGGATTTATGCCACATCCAACCTGGCTTCTGGAGTCCC  
TGTTTCGCTTCAGTGGCAGTGGGTCTGGGACTTCTTACTCTCTCACAAT  
CAGCAGAGTGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAGT  
GGACTAGTAACCCACCCACGTTTCGGAGGGGGGACCAAGCTGGAAATC  
AAA

FIG. 93B

Met Asp Phe Gln Val Gln Ile Ile Ser Phe Leu Leu Ile Ser Ala Ser Val Ile Met Ser  
Arg Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Ile Leu Ser Ala Ser Pro Gly Glu  
Lys Val Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Ile His Trp Phe Gln  
Gln Lys Pro Gly Ser Ser Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser  
Gly Val Pro Val Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile  
Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Thr Ser Asn  
Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys

332/498

FIG. 94A

ATGGGTTGGAGCCTCATCTTGCTCTTCCTTGTCGCTGTTGCTACGCGTG  
TCCTGTCCCAGGTACAACCTGCAGCAGCCTGGGGCTGAGCTGGTGAAG  
CCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCTGGCTACACATTT  
ACCAGTTACAATATGCACTGGGTAAAACAGACACCTGGTCGGGGCCT  
GGAATGGATTGGAGCTATTTATCCCGGAAATGGTGATACTTCCTACAA  
TCAGAAGTTCAAAGGCAAGGCCACATTGACTGCAGACAAATCCTCCA  
GCACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCG  
GTCTATTACTGTGCAAGATCGACTTACTACGGCGGTGACTGGTACTTC  
AATGTCTGGGGCGCAGGGACACGGTCACCGTCTCTGCA

FIG. 94B

Met Gly Trp Ser Leu Ile Leu Leu Phe Leu Val Ala Val Ala Thr Arg Val Leu Ser  
Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala Ser Val Lys  
Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met His Trp Val Lys  
Gln Thr Pro Gly Arg Gly Leu Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly Asp  
Thr Ser Tyr Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser  
Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr  
Cys Ala Arg Ser Thr Tyr Tyr Gly Gly Asp Trp Tyr Phe Asn Val Trp Gly Ala Gly  
Thr Thr Val Thr Val Ser Ala

333/498

FIG. 95A

GACGTCGCGGCCGCTCTAGGCCTCCAAAAAGCCTCCTCACTACTTCT  
GGAATAGCTCAGAGGCCGAGGCGGCCTCGGCCTCTGCATAAATAAAA  
AAAATTAGTCAGCCATGCATGGGGCGGAGAATGGGCGGAAGTGGGCG  
GAGTTAGGGGCGGGATGGGCGGAGTTAGGGGCGGGACTATGGTTGCT  
GACTAATTGAGATGCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCT  
GGGGACTTTCCACACCTGGTTGCTGACTAATTGAGATGCATGCTTTGC  
ATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCCTAACTGA  
CACACATTCCACAGAATTAATTCCCTAGTTATTAATAGTAATCAATT  
ACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCGCGTTACATAA  
CTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCC  
ATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGA  
CTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACT  
TGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACG  
TCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCT  
TATGGGACTTTTCTACTTGGCAGTACATCTACGTATTAGTCATCGCTA  
TTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGC  
GGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTTGACGTCAATG  
GGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTTCGTA  
ACAACCTCCGCCCCATTGACGCAAAATGGGCGGTAGGCGTGTACGGTGG  
GAGGTCTATATAAGCAGAGCTGGGTACGTGAACCGTCAGATCGCCTG  
GAGACGCCATCACAGATCTCTCACCATGAGGGTCCCCGCTCAGCTCCT  
GGGGCTCCTGCTGCTCTGGCTCCAGGTGCACGATGTGATGGTACCAA  
GGTGGAATCAAACGTACGGTGGCTGCACCATCTGTCTTCATCTTCCC  
GCCATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGTGTGCCT  
GCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGG  
ATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAG  
GACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAG  
CAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCC  
ATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAG  
TGTTGAATTCAGATCCGTTAACGGTTACCAACTACCTAGACTGGATTG  
GTGACAACATGCGGCCGTGATATCTACGTATGATCAGCCTCGACTGTG  
CCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCTCCCCCGTGCCTTCT  
TGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAAATGAGG  
AAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTG  
GGGTGGGGCAGGACAGCAAGGGGGGAGGATTGGGAAGACAATAGCAG  
GCATGCTGGGGATGCGGTGGGCTCTATGGAACCAGCTGGGGCTCGAC  
AGCTATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGC  
CCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTT  
GGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGT  
TTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGA  
TTTCCAAGTCTCCACCCCATTTGACGTCAATGGGAGTTTGTTTTGGCAC

334/498

FIG. 95B

CAAAATCAACGGGACTTTCCAAAATGTCGTAACAACCTCCGCCCCATTG  
ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAG  
AGCTGGGTACGTCCTCACATTCACTGATCAGCACTGAACACAGACCC  
GTCGACATGGGTTGGAGCCTCATCTTGCTCTTCCTTGTCGCTGTTGCTA  
CGCGTGTGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCCT  
CCTCCAAGAGCACCTCTGGGGGACAGCGGCCCTGGGCTGCCTGGTC  
AAGGACTACTTCCCCGAACCGGTGACGGTGTGCTGGAACCTCAGGCGC  
CCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGG  
ACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGG  
CACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCA  
AGGTGGACAAGAAAGCAGAGCCCAAATCTTGTGACAAAACCTCACACA  
TGCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTC  
CTCTTCCCCCCTAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCT  
GAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGT  
CAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGA  
CAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGC  
GTCCTCACCGTCTGCAACAGGACTGGCTGAATGGCAAGGACTACAA  
GTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCA  
TCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTG  
CCCCCATCCCGGGATGAGCTGACCAGGAACCAGGTCAGCCTGACCTG  
CCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGA  
GCAATGGGCAGCCGGAGAACAACCTACAAGACCACGCCTCCCGTGCTG  
GACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAG  
AGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGA  
GGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGG  
TAAATGAGGATCCGTTAACGGTTACCAACTACCTAGACTGGATTCTGTG  
ACAACATGCGGCCGTGATATCTACGTATGATCAGCCTCGACTGTGCCT  
TCTAGTTGCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTGCCTTCTTGA  
CCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAA  
TTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGG  
TGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCA  
TGCTGGGGATGCGGTGGGCTCTATGGAACCAGCTGGGGCTCGACAGC  
GCTGGATCTCCCGATCCCCAGCTTTGCTTCTCAATTTCTTATTGTCATA  
ATGAGAAAAAAAGGAAAATTAATTTTAACACCAATTCAGTAGTTGAT  
TGAGCAAATGCGTTGCCAAAAGGATGCTTTAGAGACAGTGTTCTCT  
GCACAGATAAGGACAAACATTATTCAGAGGGAGTACCCAGAGCTGAG  
ACTCCTAAGCCAGTGAGTGGCACAGCATTCTAGGGAGAAATATGCTT  
GTCATCACCGAAGCCTGATTCCGTAGAGCCACACCTTGGTAAGGGCC  
AATCTGCTCACACAGGATAGAGAGGGCAGGAGCCAGGGCAGAGCAT  
ATAAGGTGAGGTAGGATCAGTTGCTCCTCACATTTGCTTCTGACATAG  
TTGTGTTGGGAGCTTGGATAGCTTGGACAGCTCAGG

335/498

FIG. 95C

GCTGCGATTTTCGCGCCAAACTTGACGGCAATCCTAGCGTGAAGGCTG  
GTAGGATTTTATCCCCGCTGCCATCATGGTTCGACCATTGAACTGCAT  
CGTCGCCGTGTCCCAAATATGGGGATTGGCAAGAACGGAGACCTAC  
CCTGGCCTCCGCTCAGGAACGAGTTCAAGTACTTCCAAAGAATGACC  
ACAACCTCTTCAGTGGAAGGTAAACAGAATCTGGTGATTATGGGTAG  
GAAAACCTGGTTCTCCATTCTGAGAACAAATCGACCTTTAAAGGACA  
GAATTAATATAGTTCTCAGTAGAGAACTCAAAGAACCACCACGAGGA  
GCTCATTTTCTTGCCAAAAGTTTGGATGATGCCTTAAGACTTATTGAA  
CAACCGGAATTGGCAAGTAAAGTAGACATGGTTTGGATAGTCGGAGG  
CAGTTCTGTTTACCAGGAAGCCATGAATCAACCAGGCCACCTTAGACT  
CTTTGTGACAAGGATCATGCAGGAATTTGAAAGTGACACGTTTTTCCC  
AGAAATTGATTTGGGGAAATATAAACTTCTCCCAGAATACCCAGGCG  
TCCTCTCTGAGGTCCAGGAGGAAAAAGGCATCAAGTATAAGTTTGAA  
GTCTACGAGAAGAAAGACTAACAGGAAGATGCTTTCAAGTTCTCTGC  
TCCCCTCCTAAAGTCATGCATTTTTATAAGACCATGGGACTTTTGCTG  
GCTTTAGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGT  
TTGCCCCCTCCCCCGTGCCCTTCTTGACCCTGGAAGGTGCCACTCCCAC  
TGTCCTTTCCTAATAAAAATGAGGAAATTGCATCGCATTGTCTGAGTAG  
GTGTCATTCTATTCTGGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGG  
AGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCT  
ATGGAACCAGCTGGGGCTCGAGCTACTAGCTTTGCTTCTCAATTTCTT  
ATTTGCATAATGAGAAAAAAAGGAAAATTAATTTTAACACCAATTCA  
GTAGTTGATTGAGCAAATGCGTTGCCAAAAGGATGCTTTAGAGACA  
GTGTTCTCTGCACAGATAAGGACAAACATTATTCAGAGGGGAGTACCC  
AGAGCTGAGACTCCTAAGCCAGTGAGTGGCACAGCATTCTAGGGAGA  
AATATGCTTGTCATCACCGAAGCCTGATTCCGTAGAGCCACACCTTGG  
TAAGGGCCAATCTGCTCACACAGGATAGAGAGGGCAGGAGCCAGGG  
CAGAGCATATAAGGTGAGGTAGGATCAGTTGCTCCTCACATTTGCTTC  
TGACATAGTTGTGTTGGGAGCTTGATCGATCCTCTATGGTTGAACAA  
GATGGATTGCACGCAGGTTCTCGGGCCGCTTGGGTGGAGAGGCTATTC  
GGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGT  
GTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTGTCAAGACCGA  
CCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTAT  
CGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG  
TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGG  
CAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATC  
ATGGCTGATGCAATGCGGGCGGCTGCATACGCTTGATCCGGCTACCTGC  
CCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCG  
GATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATC  
AGGGGCTCGCGCCAGCCGAACGTTCGCCAGGCTCAAGGCGCGCATG  
CCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCG

336/498

FIG. 95D

AATATCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGC  
CGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCG  
TGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGT  
GCTTTACGGTATCGCCGCTTCCCGATTTCGCAGCGCATCGCCTTCTATC  
GCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGAC  
CGACCAAGCGACGCCAACCTGCCATCACGAGATTTTCGATTCCACCG  
CCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCG  
GCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCC  
ACCCCAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATA  
GCATCACAAATTTACAAATAAAGCATTTTTTCACTGCATTCTAGTT  
GTGGTTTGTCCAACTCATCAATCTATCTTATCATGTCTGGATCGCGG  
CCGCGATCCCGTCGAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCC  
TGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGG  
AGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCAC  
ATTAATTGCGTTGCGCTCACTGCCCGCTTTCAGTCGGGAAACCTGTC  
GTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT  
TGCGTATTGGGCGCTCTTCCGCTTCCCTCGCTCACTGACTCGCTGCGCTC  
GGTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAA  
TACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGA  
GCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC  
TGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATC  
GACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATAC  
CAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACC  
CTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTG  
GCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTC  
GTTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGAC  
CGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGA  
CACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAG  
AGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA  
ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGA  
AGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAA  
CAAACCAACCGCTGGTAGCGGTGGTTTTTTTGTGTTGCAAGCAGCAGATT  
ACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTAC  
GGGGTCTGACGCTCAGTGGAACGAAAACCTCACGTAAAGGGATTTTGG  
TCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTA  
AATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTG  
ACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTC  
TATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTAC  
GATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGC  
GAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCA  
GCCGGAAGGGCCGAGCGCAGAAAGTGGTCCTGCAACTTTATCCGCCTC  
CATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC

337/498

FIG. 95E

CAGTTAATAGTTTGC GCAACGTTGTTGCCATTGCTACAGGCATCGTGG  
TGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAAC  
GATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTT  
AGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTG  
TTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGC  
CATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCAT  
TCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAA  
TACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATC  
ATTGGAAAACGTTCTTCGGGGCGAAACTCTCAAGGATCTTACCGCTG  
TTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCA  
GCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGG  
CAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAA  
TACTCATACTCTTCCTTTTCAATATTATTGAAGCATTTATCAGGGTTA  
TTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACA  
AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCT



338/498

FIG. 96A

GACGTCGCGGCCGCTCTAGGCCTCCAAAAAGCCTCCTCACTACTTCT  
GGAATAGCTCAGAGGCCGAGGCGGCCTCGGCCTCTGCATAAATAAAA  
AAAATTAGTCAGCCATGCATGGGGCGGAGAATGGGCGGAAGTGGGCG  
GAGTTAGGGGCGGGATGGGCGGAGTTAGGGGCGGGACTATGGTTGCT  
GACTAATTGAGATGCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCT  
GGGGACTTTCCACACCTGGTTGCTGACTAATTGAGATGCATGCTTTGC  
ATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCCTAACTGA  
CACACATTCCACAGAATTAATCCCCTAGTTATTAATAGTAATCAATT  
ACGGGGTCAATAGTTCATAGCCCATATATGGAGTTCGCGTTACATAA  
CTTACGGTAAATGGCCCCGCCTGGCTGACCGCCCAACGACCCCCGCCC  
ATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGA  
CTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCCACT  
TGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACG  
TCAATGACGGTAAATGGCCCCGCCTGGCATTATGCCCAGTACATGACCT  
TATGGGACTTTCTACTTGGCAGTACATCTACGTATTAGTCATCGCTA  
TTACCATGGTGATGCGGTTTTTGGCAGTACATCAATGGGCGTGGATACC  
GGTTTGACTCACGCGGATTTCCAAGTCTCCACCCCATTGACGTCAATG  
GGAGTTTGTGGTGGCACCAAAATCAACGGGACTTTCCAATAATGTCGTA  
ACAACCTCCGCCCCATTGACGCAATGGGCGGTAGGCGTGTACGGTGG  
GAGGTCTATATAAGCAGAGCTGGGTACGTGAACCGTCAGATCGCCTG  
GAGACGCCATCACAGATCTCTCACTATGGATTTTCAGGTGCAGATTAT  
CAGCTTCCTGCTAATCAGTGCTTCAGTCATAATGTCCAGAGGACAAAT  
TGTTCTCTCCAGTCTCCAGCAATCCTGTCTGCATCTCCAGGGGAGAA  
GGTCACAATGACTTGCAGGGCCAGCTCAAGTGTAAGTTACATCCACT  
GGTTCCAGCAGAAGCCAGGATCCTCCCCCAAACCCTGGATTTATGCCA  
CATCCAACCTGGCTTCTGGAGTCCCTGTTCGCTTCAGTGGCAGTGGGT  
CTGGGACTTCTTACTCTCTCACAATCAGCAGAGTGGAGGCTGAAGATG  
CTGCCACTTATTACTGCCAGCAGTGGACTAGTAACCCACCCACGTTTCG  
GAGGGGGGACCAAGCTGGAAATCAAACGTACGGTGGCTGCACCATCT  
GTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTACC  
TCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTA  
CAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAG  
TGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCA  
CCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCC  
TGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTT  
CAACAGGGGAGAGTGTTGAATTCAGATCCGTTAACGGTTACCAACTA  
CCTAGACTGGATTTCGTGACAACATGCGGGCCGTGATATCTACGTATGAT  
CAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGGCCCTC  
CCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCC

339/498

FIG. 96B

TAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCT  
ATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGG  
AAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGAACCA  
GCTGGGGCTCGACAGCTATGCCAAGTACGCCCCCTATTGACGTCAATG  
ACGGTAAATGGCCCGCTGGCATTATGCCCAGTACATGACCTTATGGG  
ACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCAT  
GGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTG  
ACTCACGGGGATTTCCAAGTCTCCACCCCATTTGACGTCAATGGGAGTT  
TGTTTTGGCACCAAAATCAACGGGACTTTCCAATAATGTCGTAACAAC  
CCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTC  
TATAAAGCAGAGCTGGGTACGTCCTCACATTCAGTGATCAGCACTGA  
ACACAGACCCGTCGACATGGGTGGAGCCTCATCTTGCTCTTCCTTGT  
CGCTGTTGCTACGCGTGTCTGTCCCAGGTACAACGTCAGCAGCCTGG  
GGCTGAGCTGGTGAAGCCTGGGGCCTCAGTGAAGATGTCCTGCAAGG  
CTTCTGGCTACACATTTACCAGTTACAATATGCACTGGGTAAAACAGA  
CACCTGGTCCGGGGCCTGGAATGGATTGGAGCTATTTATCCCGGAAAT  
GGTGATACTTCTTACAATCAGAAGTTCAAAGGCAAGGCCACATTGAC  
TGCAGACAAATCCTCCAGCACAGCCTACATGCAGCTCAGCAGCCTGA  
CATCTGAGGACTCTGCGGTCTATTACTGTGCAAGATCGACTTACTACG  
GCGGTGACTGGTACTTCAATGTCTGGGGCGCAGGGACCACGGTCACC  
GTCTCTGCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCC  
TCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGT  
CAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCG  
CCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAG  
GACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGG  
GCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACC  
AAGGTGGACAAGAAAGCAGAGCCCAAATCTTGTGACAAAACCTCACAC  
ATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTT  
CCTCTTCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCC  
TGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGG  
TCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAG  
ACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAG  
CGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACA  
AGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCCATCGAGAAAACC  
ATCTCCAAGCCAAAGGGCAGCCCGGAGAACCACAGGTGTACACCCT  
GCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCT  
GCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAG  
AGCAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCT  
GGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAA  
GAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATG  
AGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG  
GTAAATGAGGATCCGTTAACGGTTACCAACTACCTAGACTGGATTCTGT

340/498

FIG. 96C

GACAACATGCGGCCGTGATATCTACGTATGATCAGCCTCGACTGTGCC  
TTCTAGTTGCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTGCCTTCCTTG  
ACCCTGGAAGGTGCCACTCCCAGTGTCTTTTCTAATAAAATGAGGAA  
ATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGG  
GTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGC  
ATGCTGGGGATGCGGTGGGCTCTATGGAACCAGCTGGGGCTCGACAG  
CGCTGGATCTCCCGATCCCCAGCTTTGCTTCTCAATTTCTTATTTGCAT  
AATGAGAAAAAAGGAAAATTAATTTTAAACACCAATTCAGTAGTTGA  
TTGAGCAAATGCGTTGCCAAAAAGGATGCTTTAGAGACAGTGTTCTCT  
GCACAGATAAGGACAAACATTATTCAGAGGGAGTACCCAGAGCTGAG  
ACTCCTAAGCCAGTGAGTGGCACAGCATTCTAGGGAGAAATATGCTT  
GTCATCACCGAAGCCTGATTCCGTAGAGCCACACCTTGGTAAGGGCC  
AATCTGCTCACACAGGATAGAGAGGGCAGGAGCCAGGGCAGAGCAT  
ATAAGGTGAGGTAGGATCAGTTGCTCCTCACATTTGCTTCTGACATAG  
TTGTGTTGGGAGCTTGGATAGCTTGGACAGCTCAGGGCTGCGATTTG  
CGCCAACTTGACGGCAATCCTAGCGTGAAGGCTGGTAGGATTTTATC  
CCCGCTGCCATCATGGTTCGACCATTGAACTGCATCGTCGCCGTGTCC  
CAAAATATGGGGATTGGCAAGAACGGAGACCTACCCTGGCCTCCGCT  
CAGGAACGAGTTCAAGTACTTCCAAAGAATGACCACAACCTCTTCAG  
TGGAAGGTAAACAGAATCTGGTGATTATGGGTAGGAAAACCTGGTTC  
TCCATTCTGAGAAGAATCGACCTTTAAAGGACAGAATTAATATAGTT  
CTCAGTAGAGAACTCAAAGAACCACCACGAGGAGCTCATTTTCTTGC  
CAAAAGTTTGGATGATGCCTTAAGACTTATTGAACAACCGGAATTGG  
CAAGTAAAGTAGACATGGTTTGGATAGTCGGAGGCAGTTCTGTTTACC  
AGGAAGCCATGAATCAACCAGGCCACCTTAGACTCTTTGTGACAAGG  
ATCATGCAGGAATTTGAAAGTGACACGTTTTTCCCAGAAATTGATTTG  
GGGAAATATAAACTTCTCCCAGAAATACCCAGGCGTCCTCTCTGA  
GGTCCAGGAGGAAAAAGGCATCAAGTATAAGTTTGAAGTCTACGAGA  
AGAAAGACTAACAGGAAGATGCTTTCAGTTCTCTGCTCCCCTCCTAA  
AGCTATGCATTTTTATAAGACCATGGGACTTTTGCTGGCTTTAGATCA  
GCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCCTCCC  
CCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCAGTGTCTTTCTA  
ATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTAT  
TCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA  
GACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGAACCAGC  
TGGGGCTCGAGCTACTAGCTTTGCTTCTCAATTTCTTATTTGCATAATG  
AGAAAAAAGGAAAATTAATTTTAAACACCAATTCAGTAGTTGATTGA  
GCAAATGCGTTGCCAAAAAGGATGCTTTAGAGACAGTGTTCTCTGCA  
CAGATAAGGACAAACATTATTCAGAGGGAGTACCCAGAGCTGAGACT  
CCTAAGCCAGTGAGTGGCACAGCATTCTAGGGAGAAATATGCTTGTC  
ATCACCGAAGCCTGATTCCGTAGAGCCACACCTTGGTAAGGGCCAAT  
CTGCTCACACAGGATAGAGAGGGCAGGAGCCAGGGCAGAGCATATA  
AGGTGAGGTAGGATCAGTTGCTCCTCACATTTGCTTCTGACATAGTTG

341/498

FIG. 96D

TGTTGGGAGCTTGGATCGATCCTCTATGGTTGAACAAGATGGATTGCA  
CGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTG  
GGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTC  
AGCGCAGGGGCGCCCCGGTTCTTTTGTCAAGACCGACCTGTCCGGTGC  
CCTGAATGAACTGCAGGACGAGGCGAGCGCGGCTATCGTGGCTGGCCA  
CGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGG  
GAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTG  
TCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCA  
ATGCGGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCAC  
CAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG  
TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGC  
CAGCCGAAGTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAG  
GATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTG  
GAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTG  
GCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGA  
AGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTAT  
CGCCGCTCCCGATTTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGA  
GTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGAC  
GCCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGA  
AAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCT  
CCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCCACCCCAACTTGTT  
TATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTT  
CACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAA  
ACTCATCAATCTATCTTATCATGTCTGGATCGCGGCCGCGATCCCGTC  
GAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGT  
TATCCGCTCACAAATCCACACAACATACGAGCCGGAAGCATAAAGTG  
TAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT  
GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCA  
TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGC  
GCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTCGGCT  
GCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCA  
CAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCA  
GCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC  
ATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGT  
CAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC  
CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTAC  
CGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCA  
ATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAAGTTCGTTCTGCTCAA  
GCTGGGCTGTGTGCACGAACCCCCGTTTCAGCCCGACCGCTGCGCCTT  
ATCCGGTAAGTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC

342/498

FIG. 96E

GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATG  
TAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTAC  
ACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACC  
TTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGC  
TGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAA  
AAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGC  
TCAGTGGAACGAAAACCTCACGTAAAGGGATTTTGGTCATGAGATTATC  
AAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAA  
ATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATG  
CTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCC  
ATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGG  
CTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTC  
ACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCG  
AGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTA  
ATTGTTGCCGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGC  
GCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCGT  
TTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTA  
CATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTC  
CGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTA  
TGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCT  
TTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTA  
TGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACC  
GCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCT  
TCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCTG  
ATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCA  
CCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAA  
AAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCT  
TTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGG  
ATACATATTTGAATGTATTTAGAAAAATAAACAATAGGGGTTCCGC  
GCACATTTCCCCGAAAAGTGCCACCT

343/498

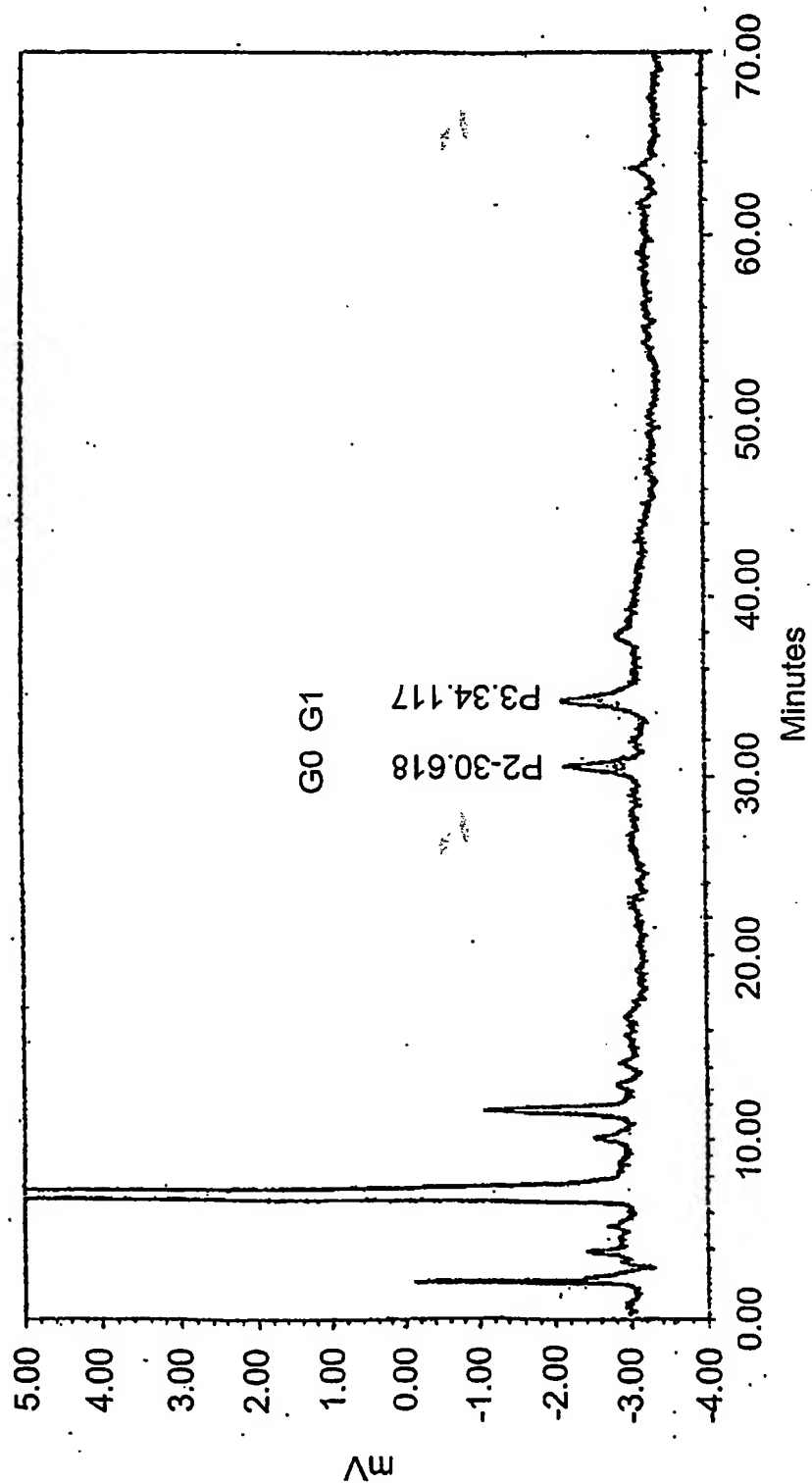


FIG. 97A

344/498

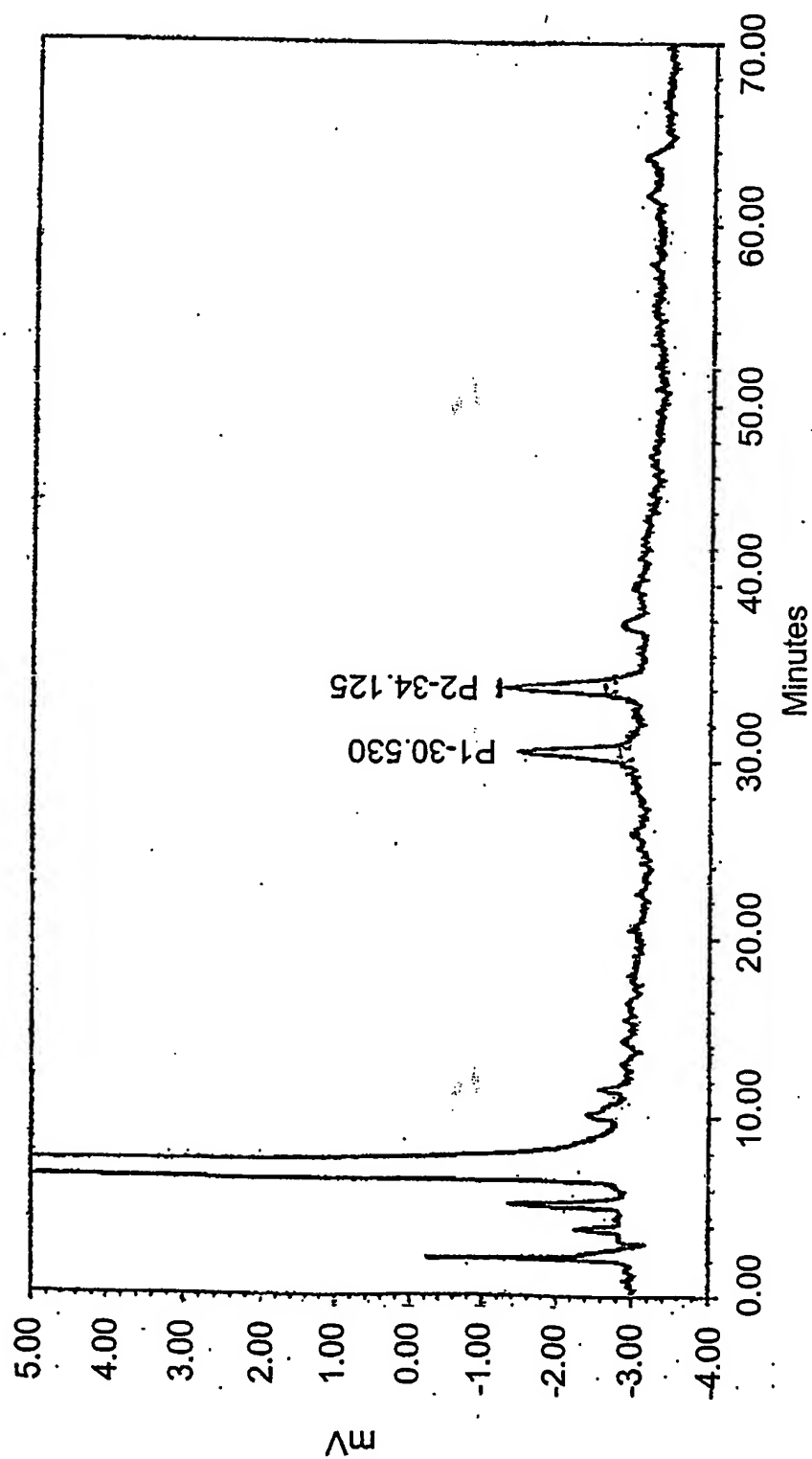


FIG. 97B

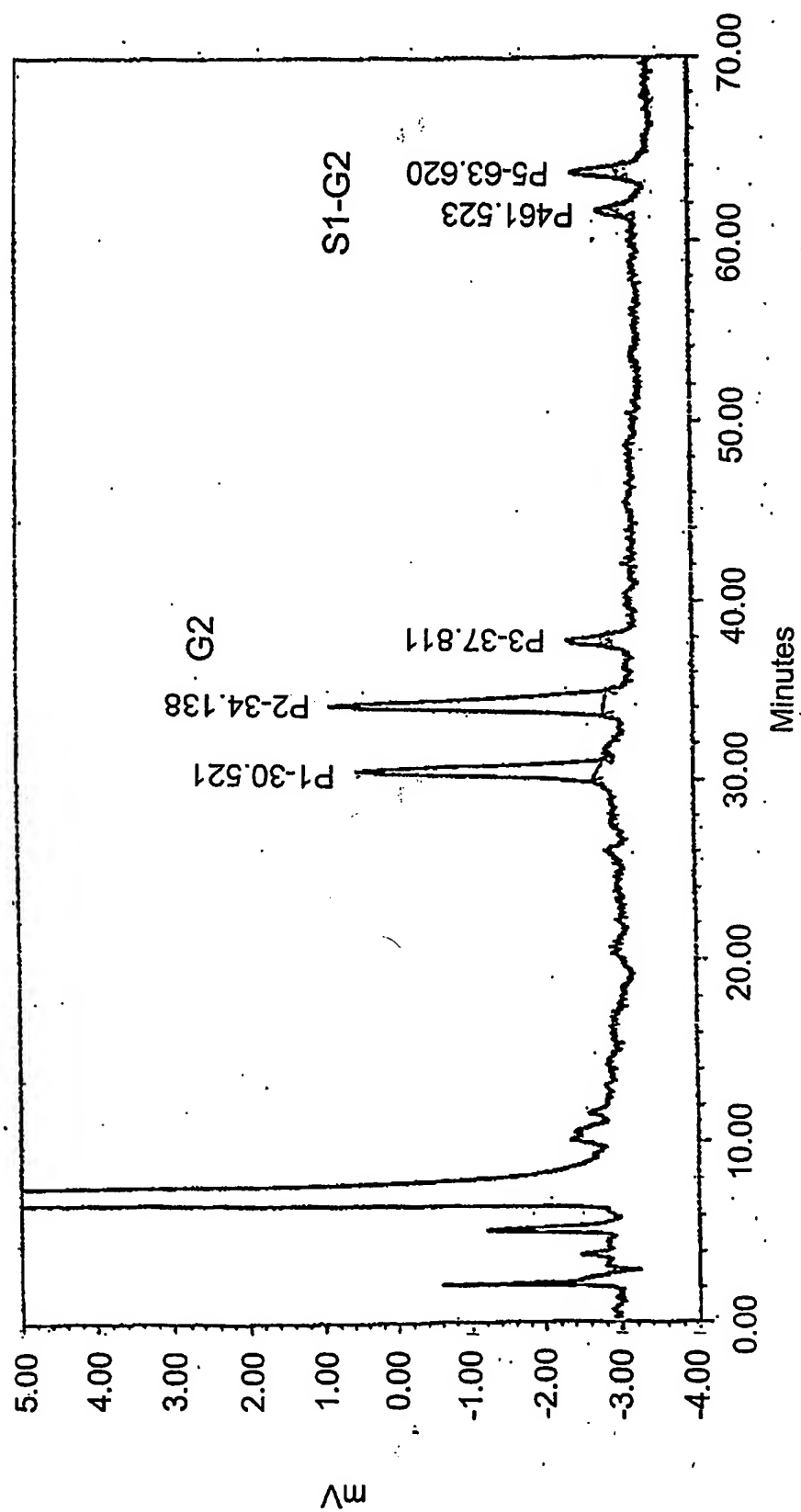


FIG. 97C



346/498

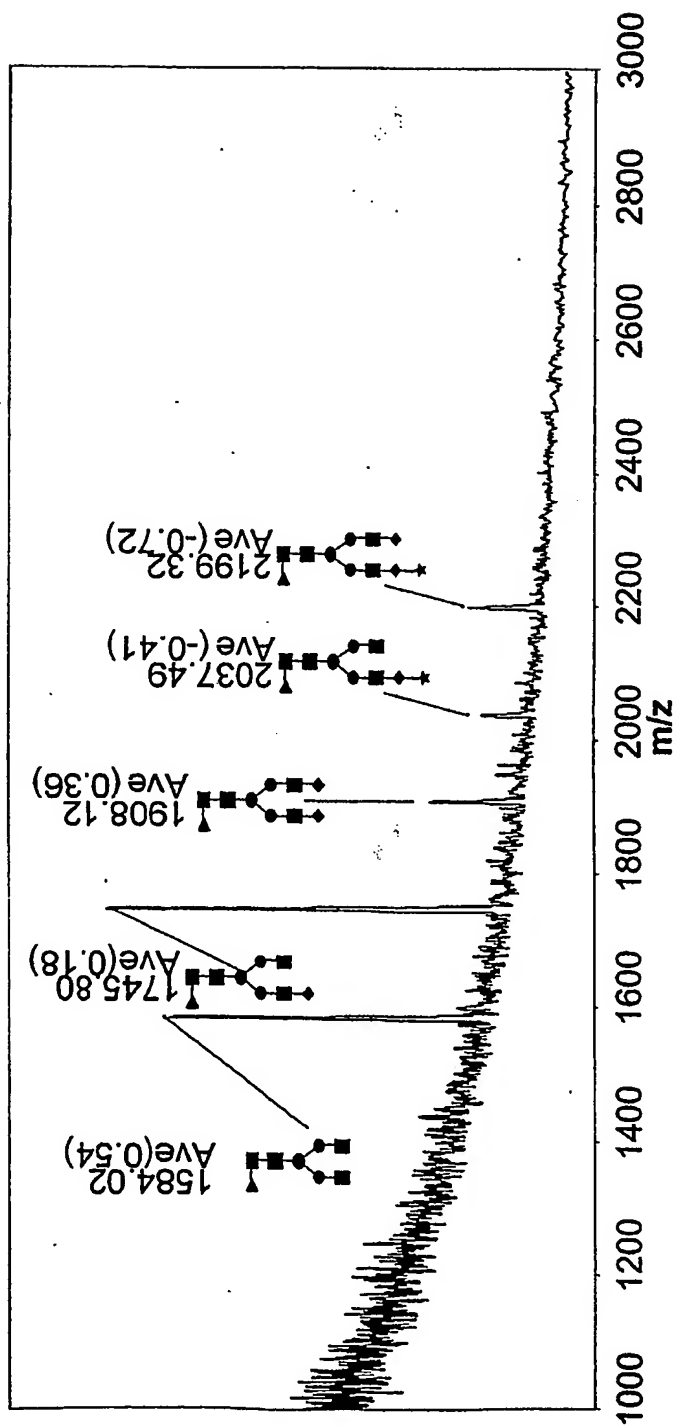


FIG. 98A

347/498

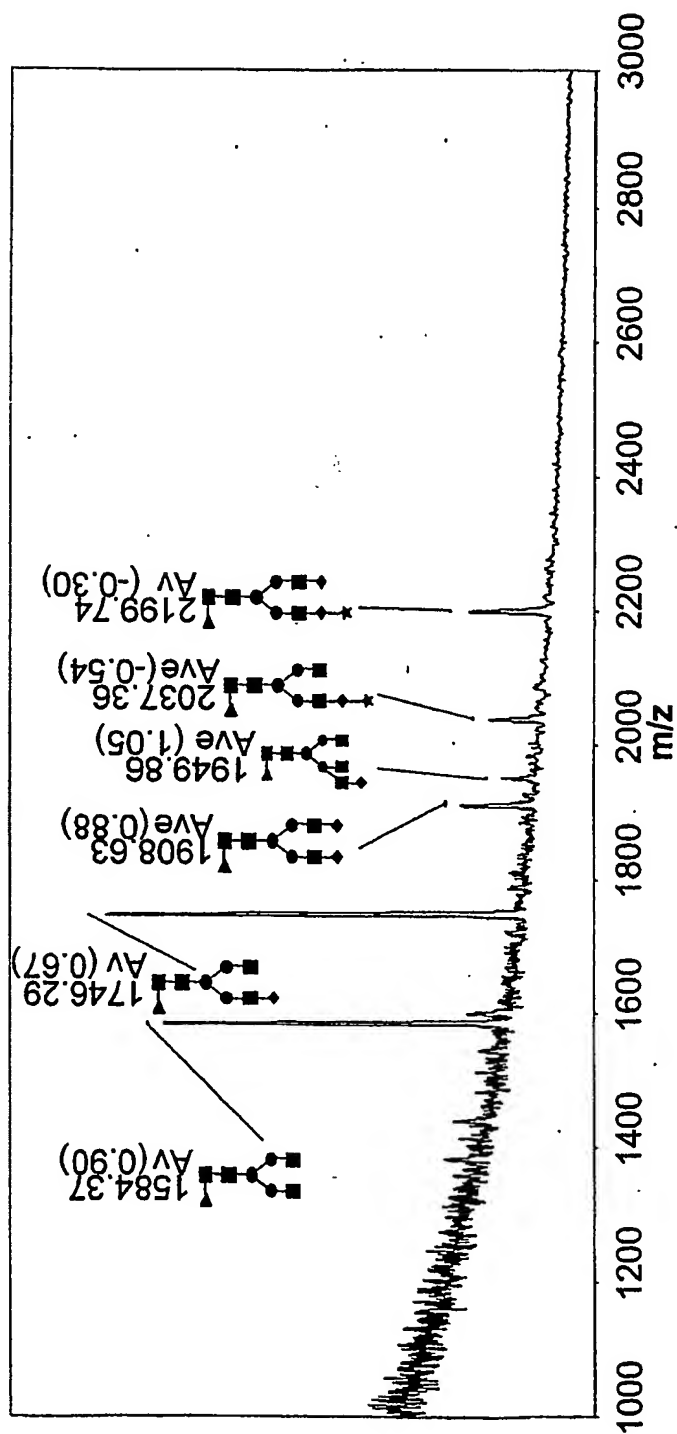


FIG. 98B

348/498

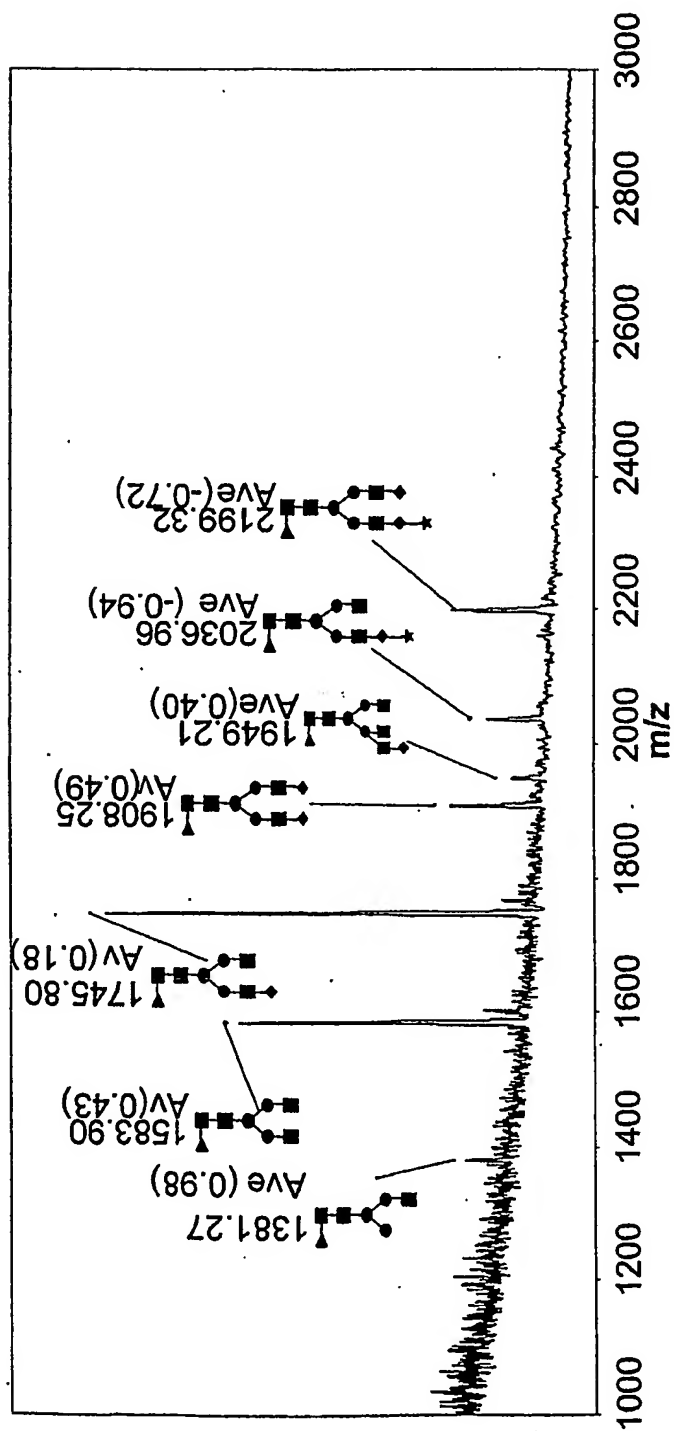


FIG. 98C

349/498

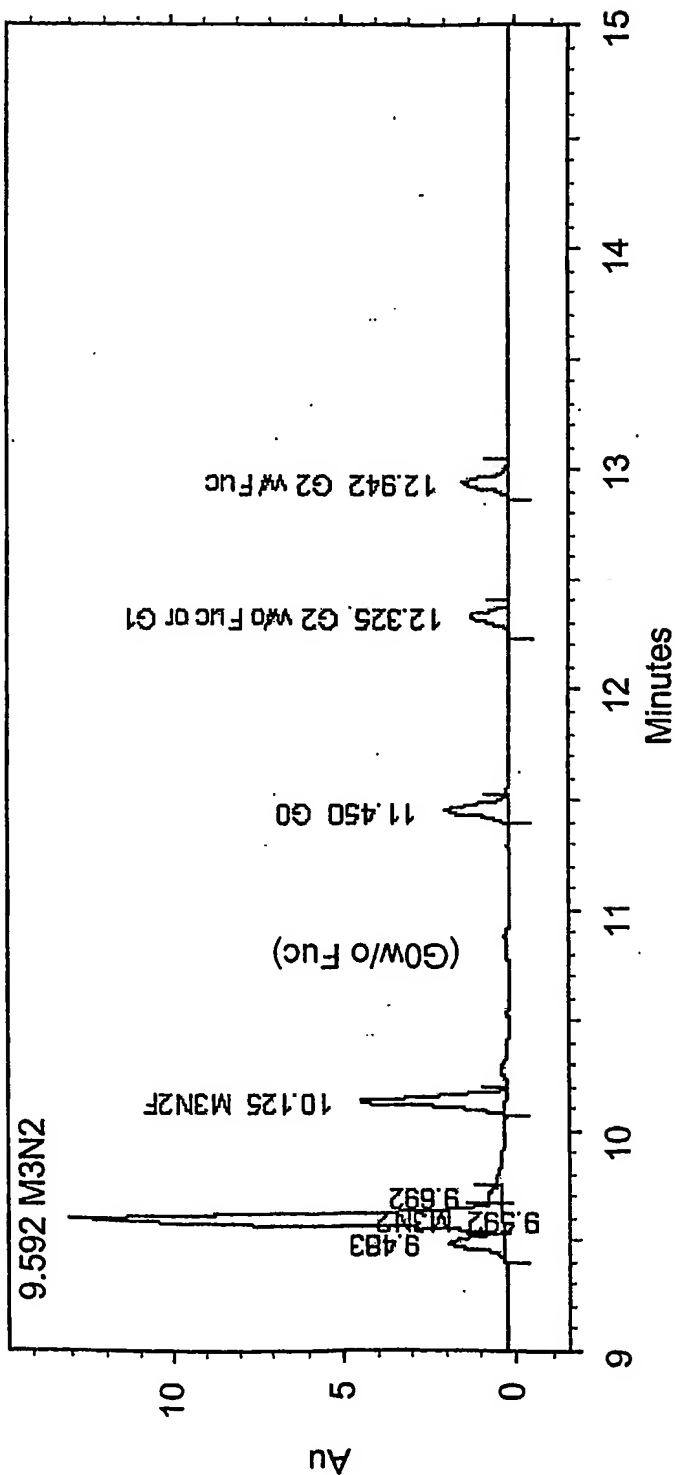


FIG. 99A

350/498

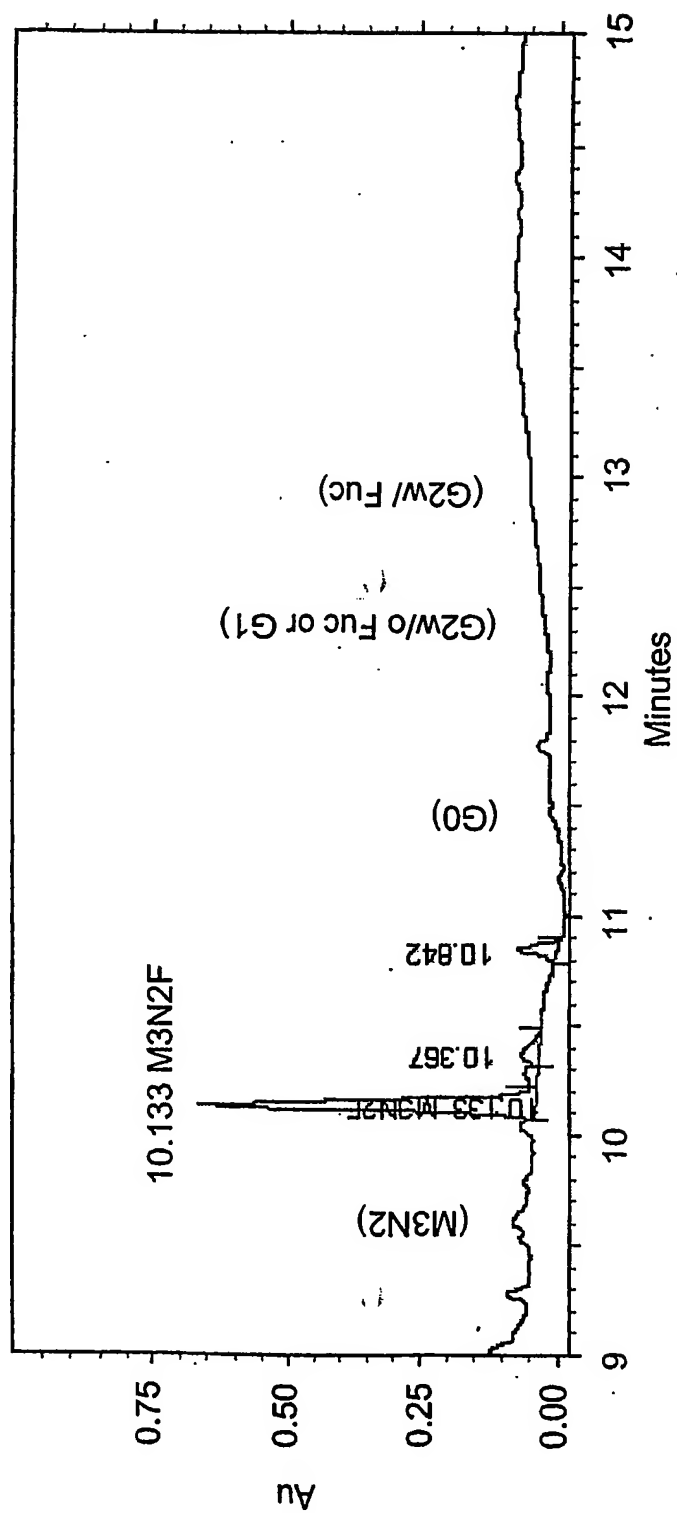


FIG. 99B

351/498

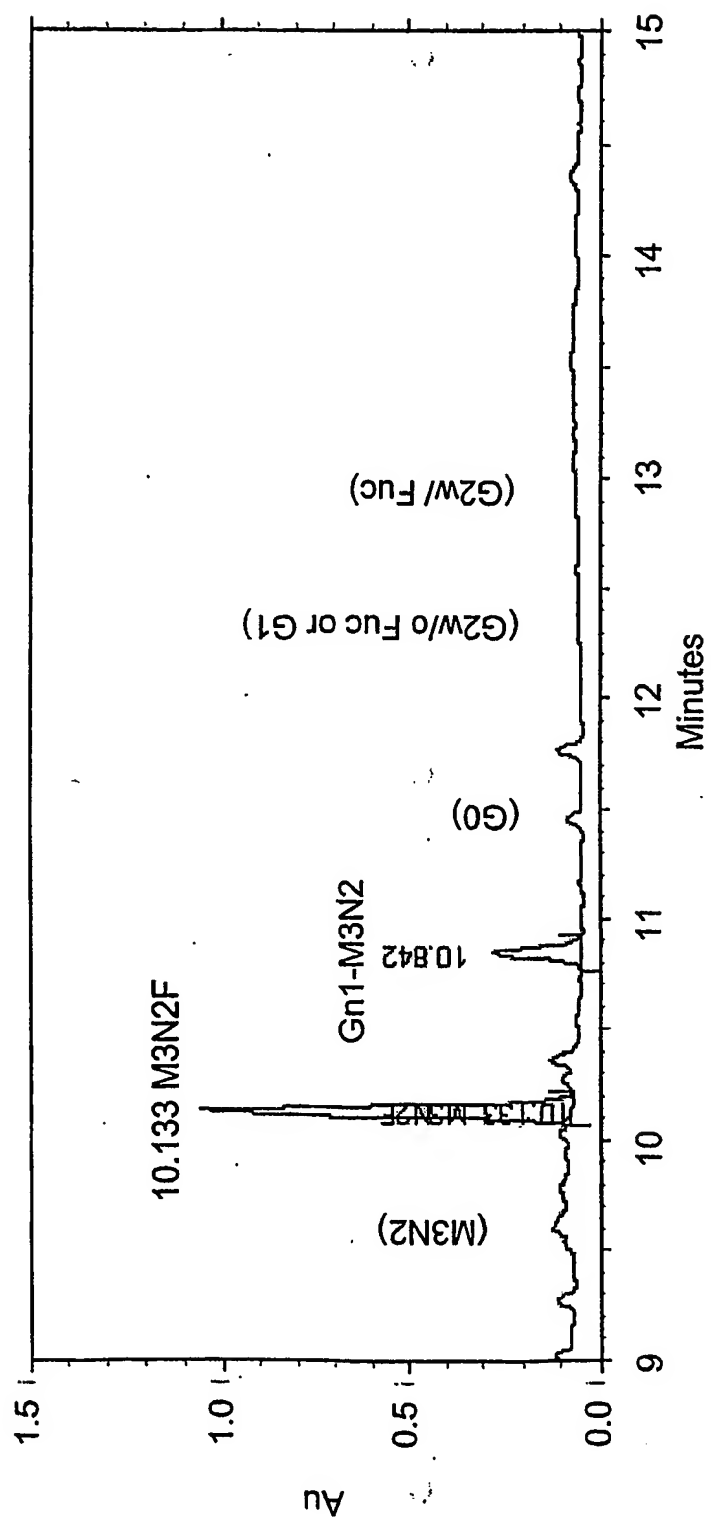


FIG. 99C

352/498

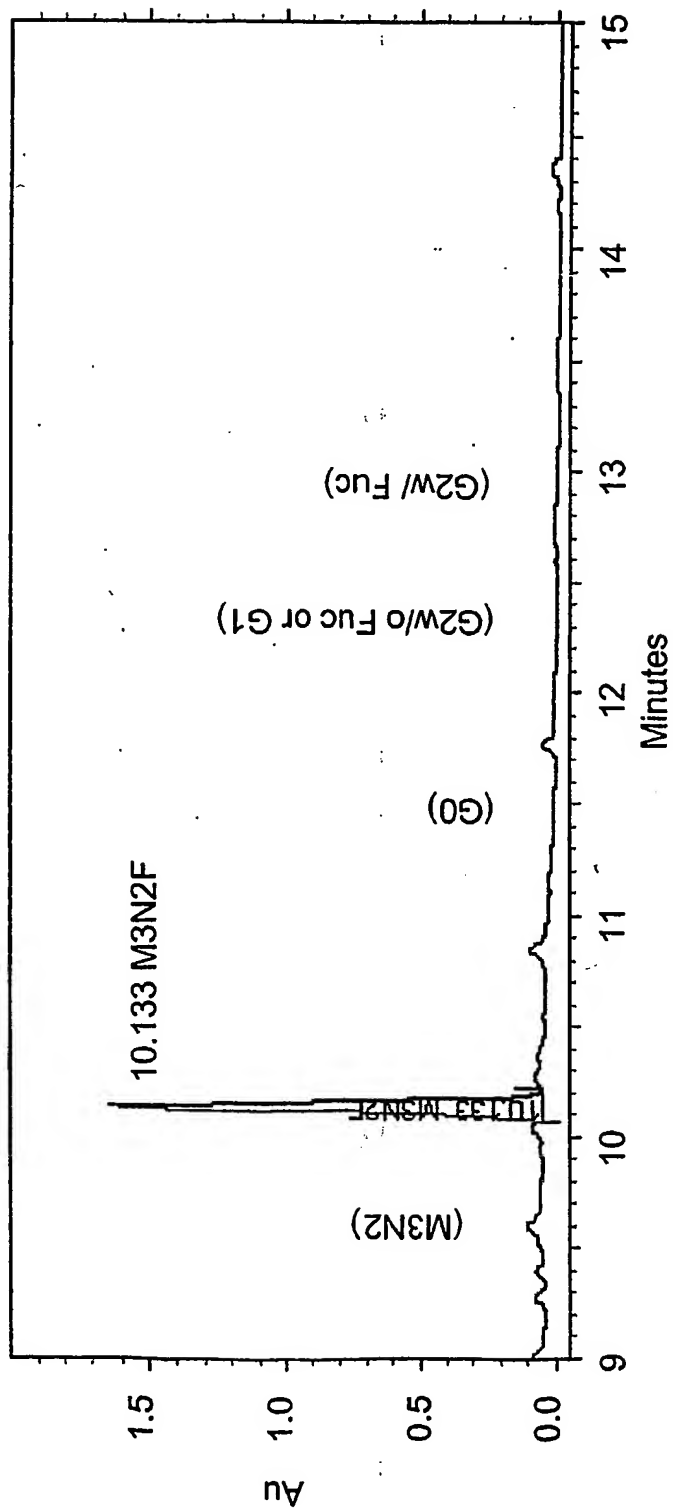


FIG. 99D

353/498

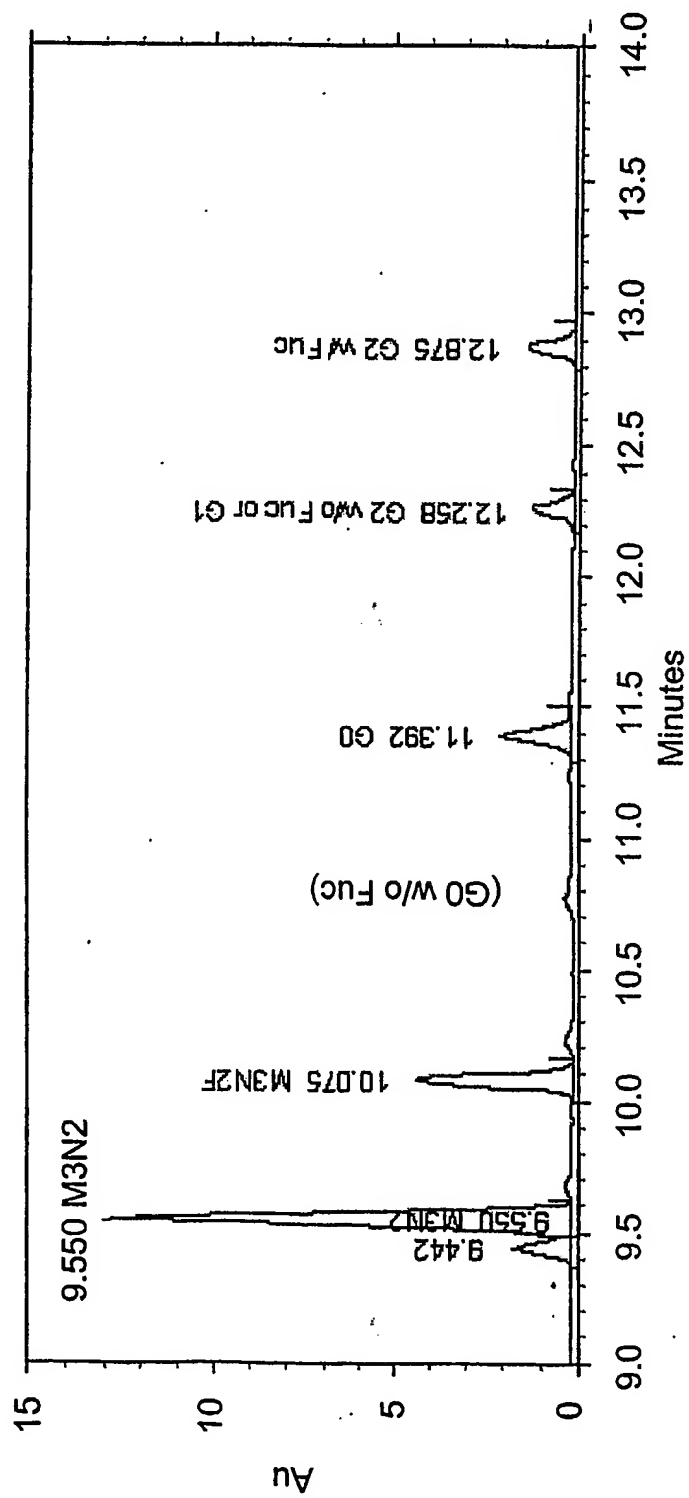


FIG. 100A



354/498

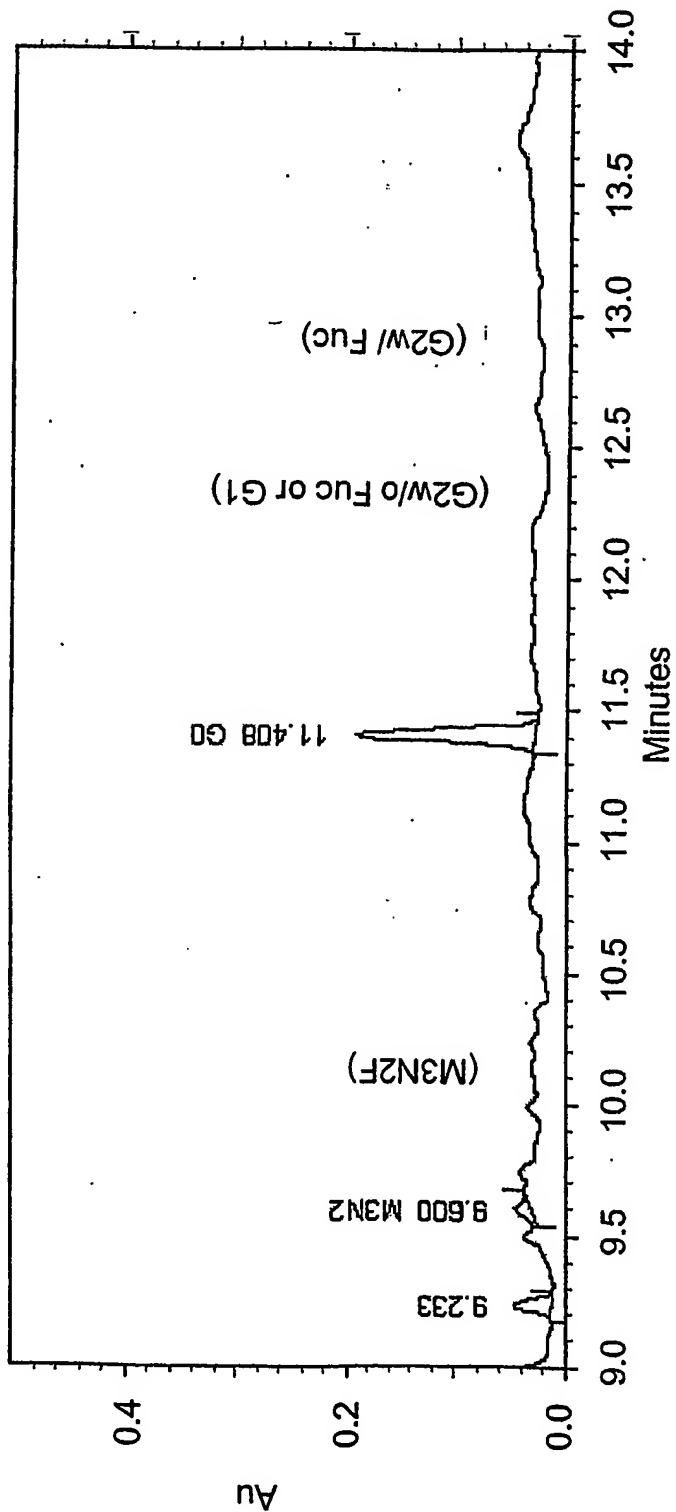


FIG. 100B

355/498

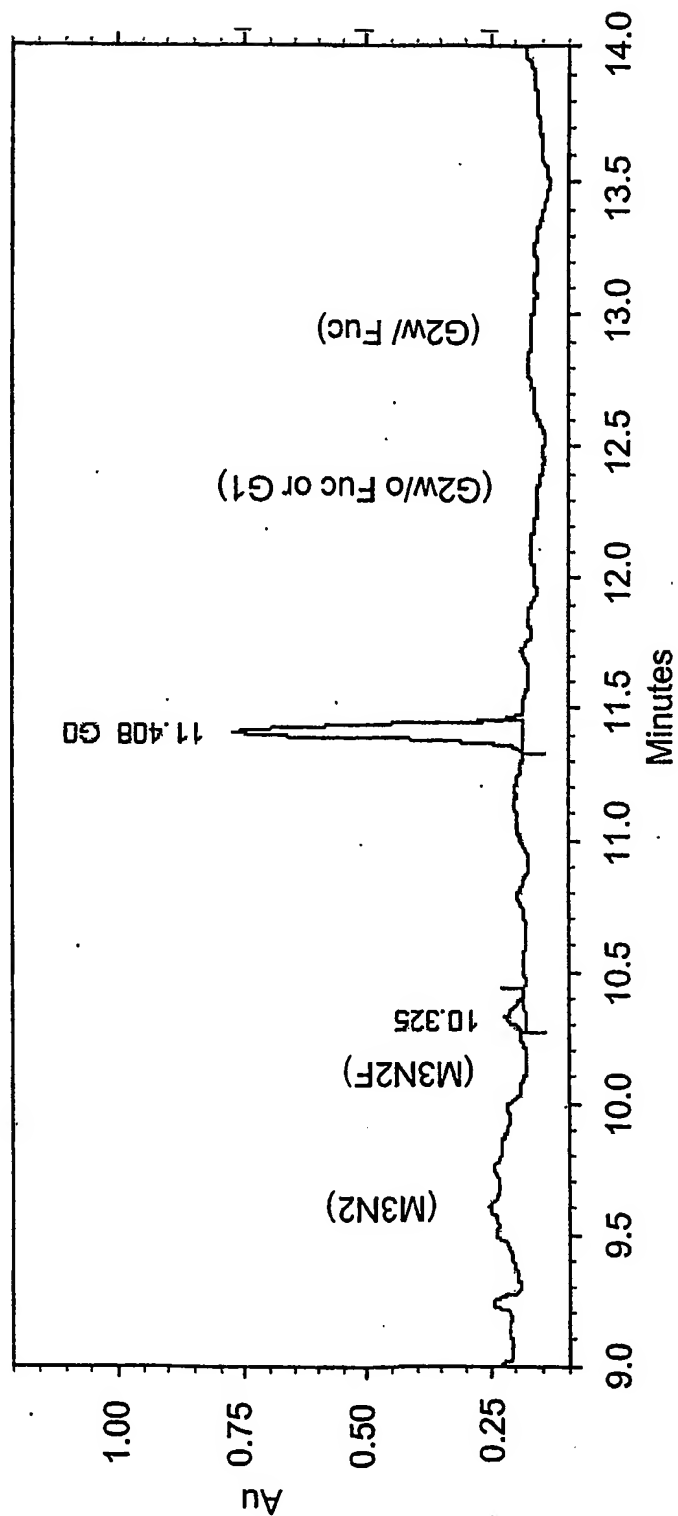


FIG. 100C

356/498

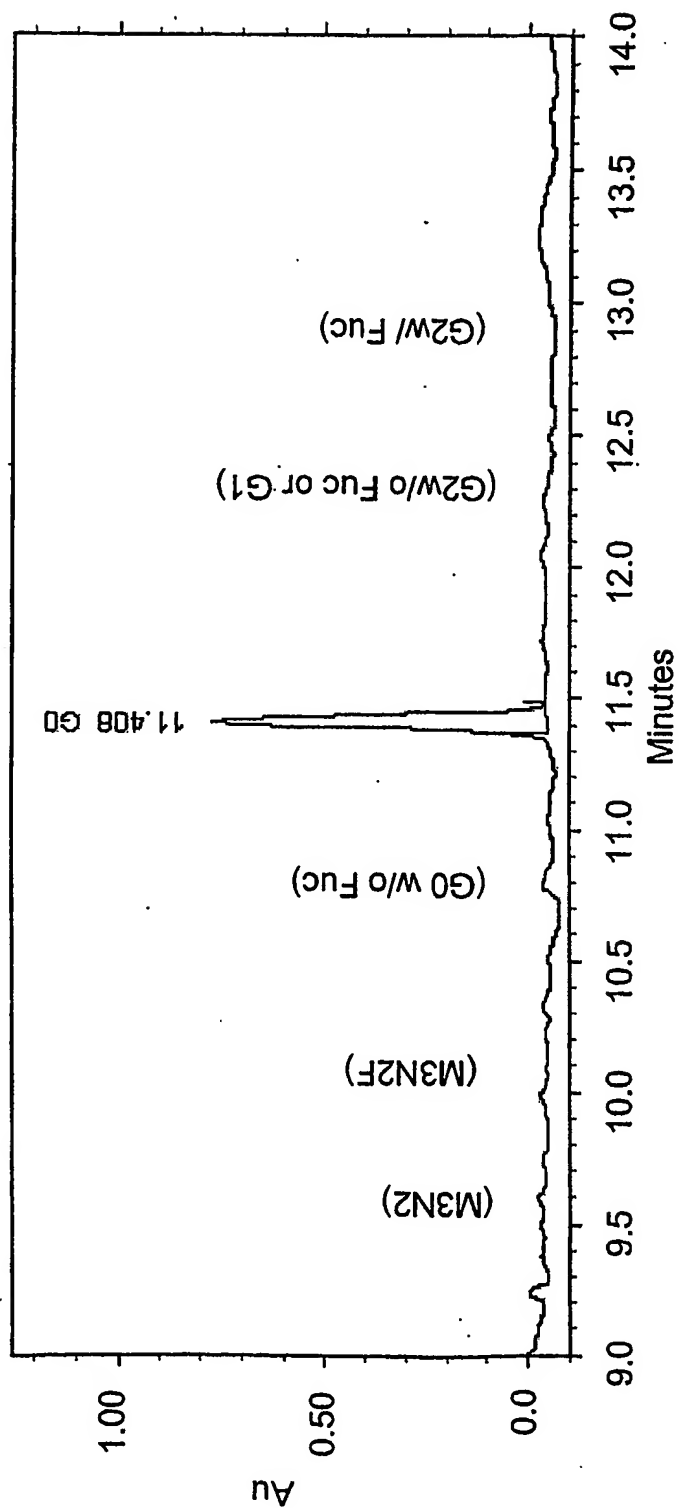


FIG. 100D

357/498

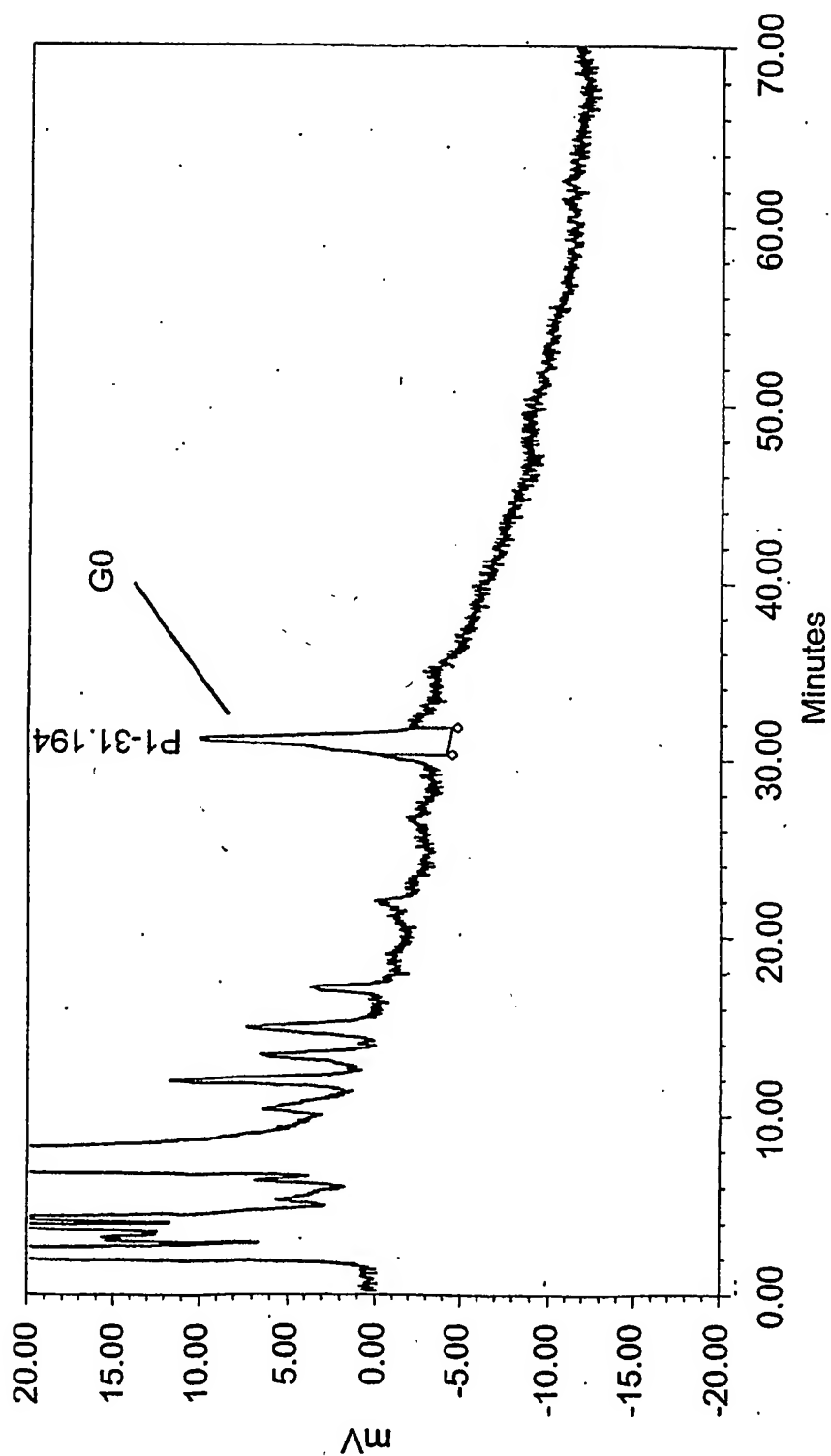


FIG. 101A

358/498

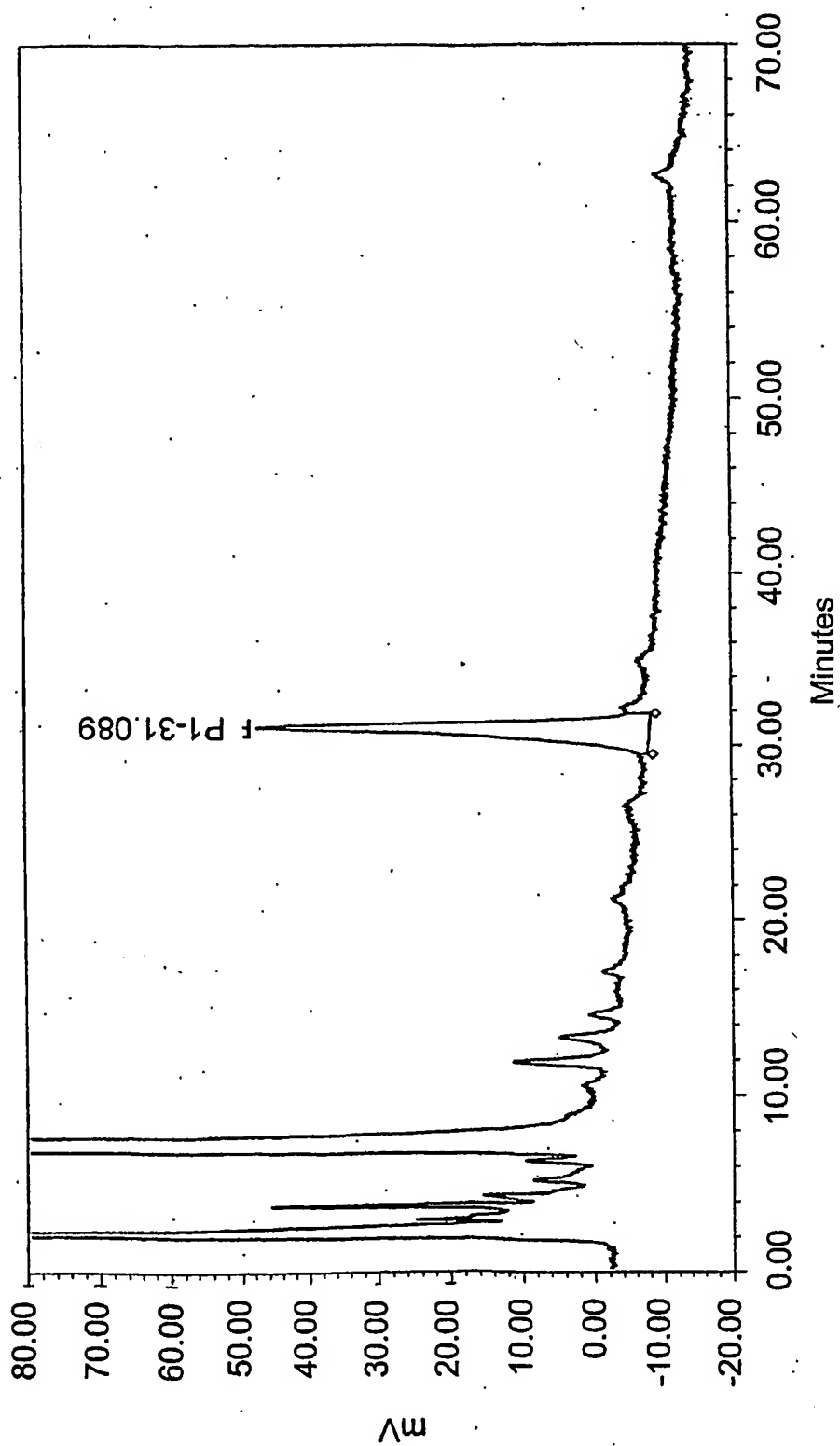


FIG. 101B

359/498

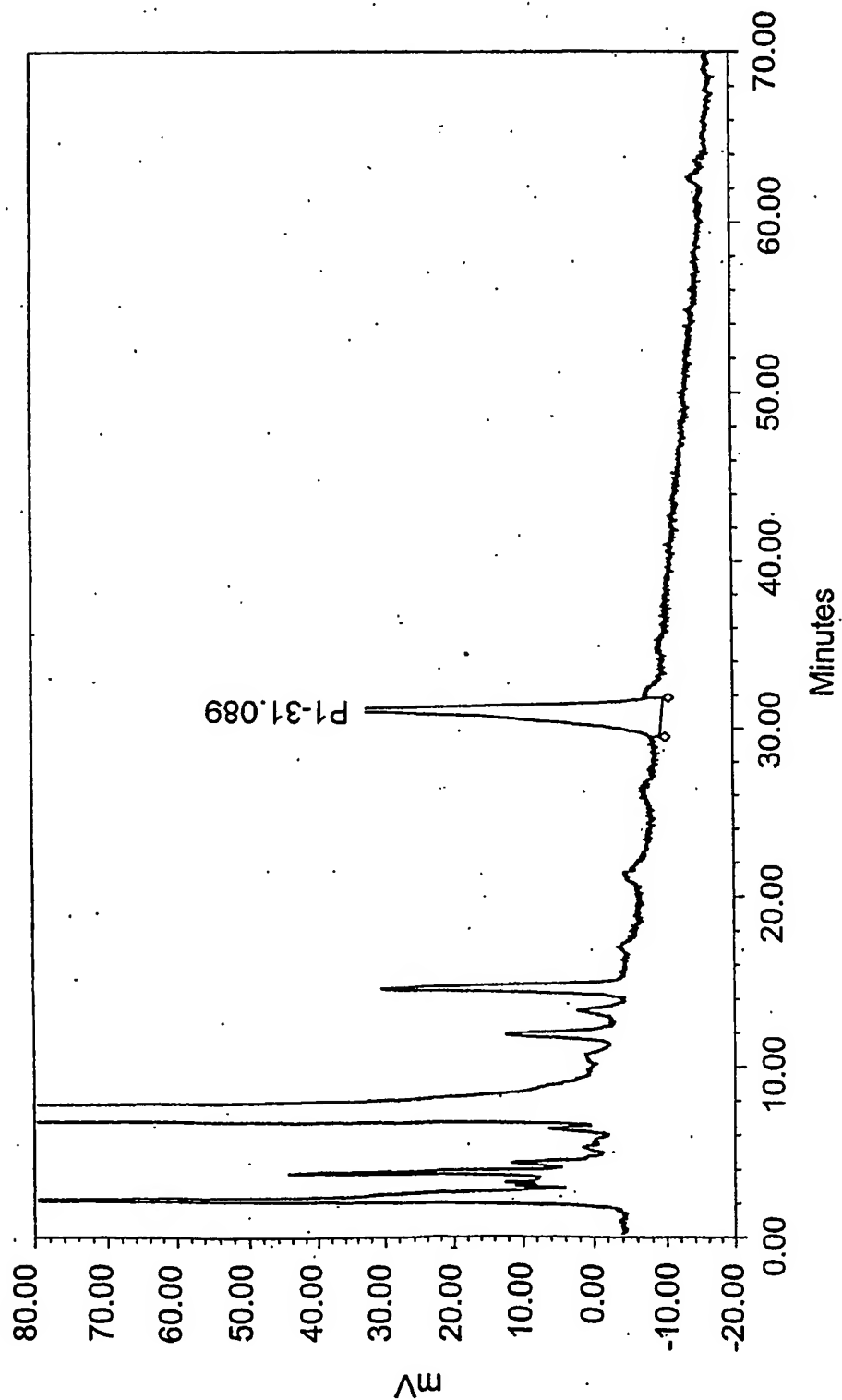


FIG. 101C

360/498

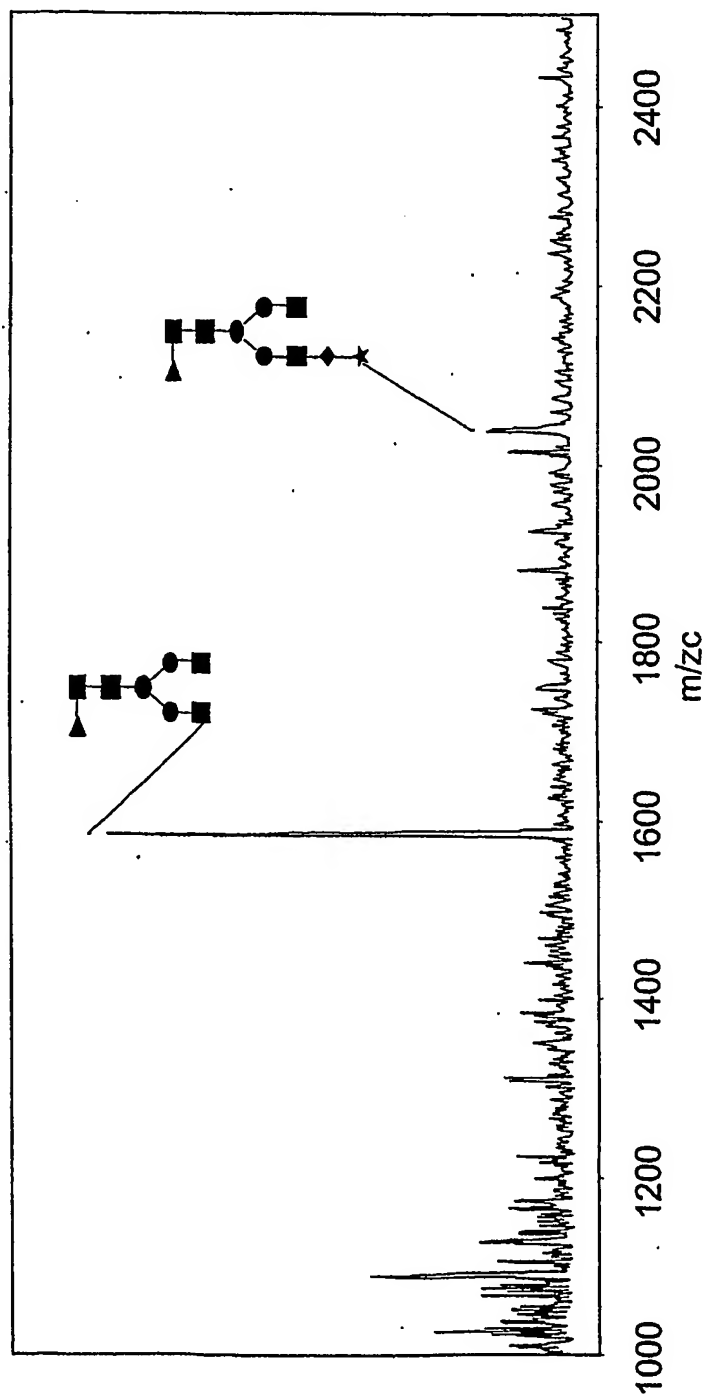


FIG. 102A

361/498

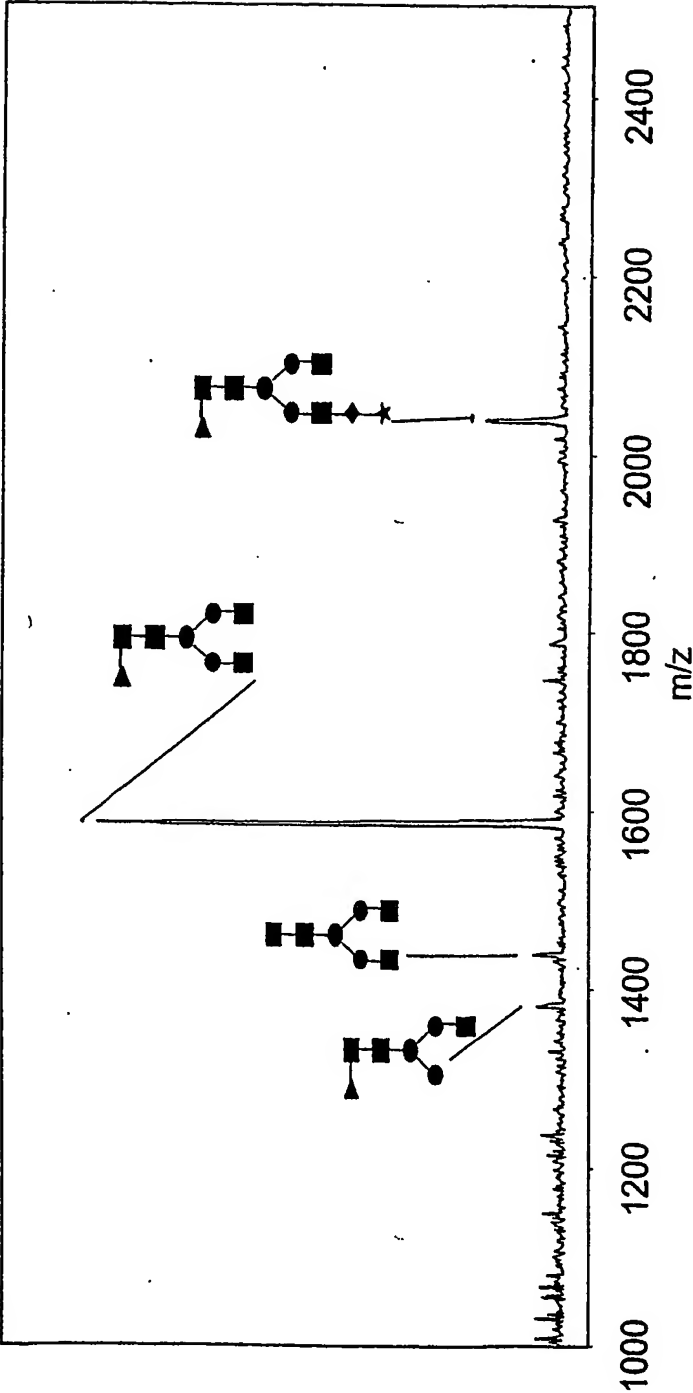


FIG. 102B



362/498

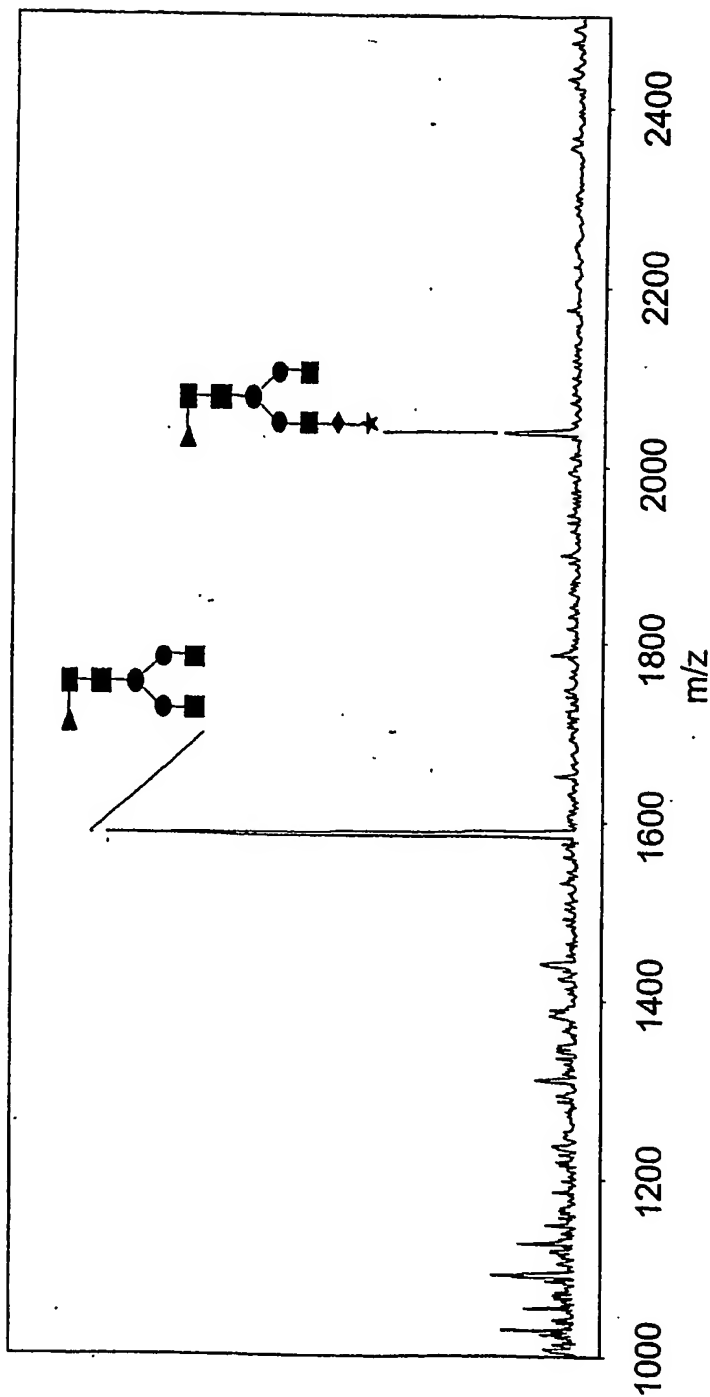


FIG. 102C

363/498

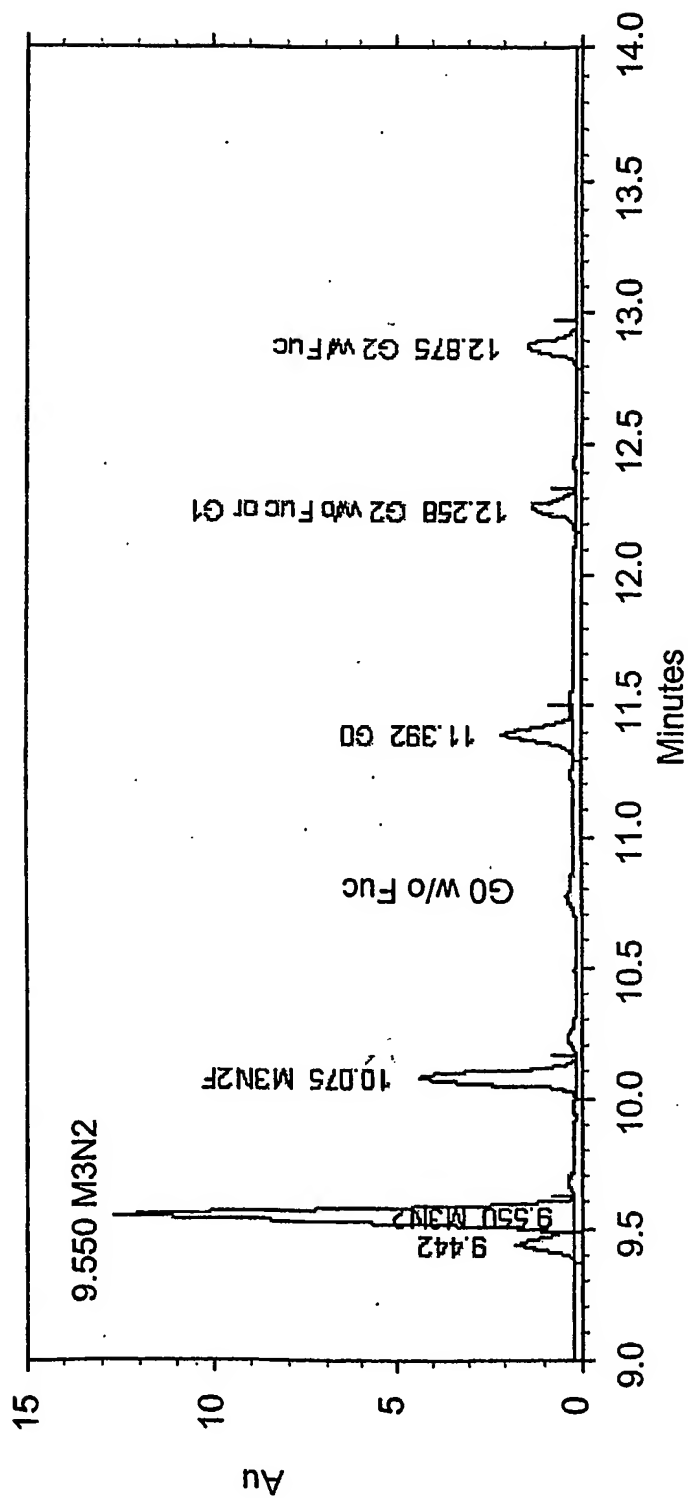


FIG. 103A

364/498

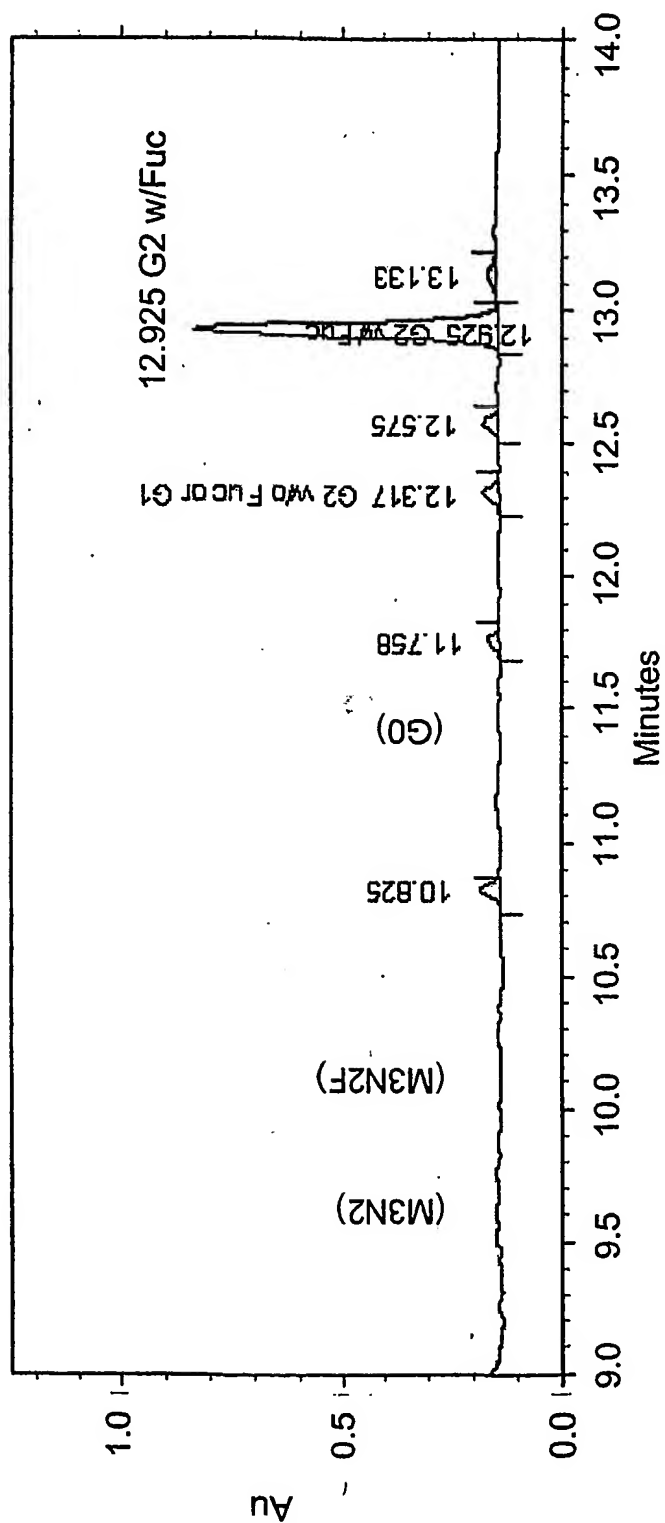


FIG. 103B

365/498

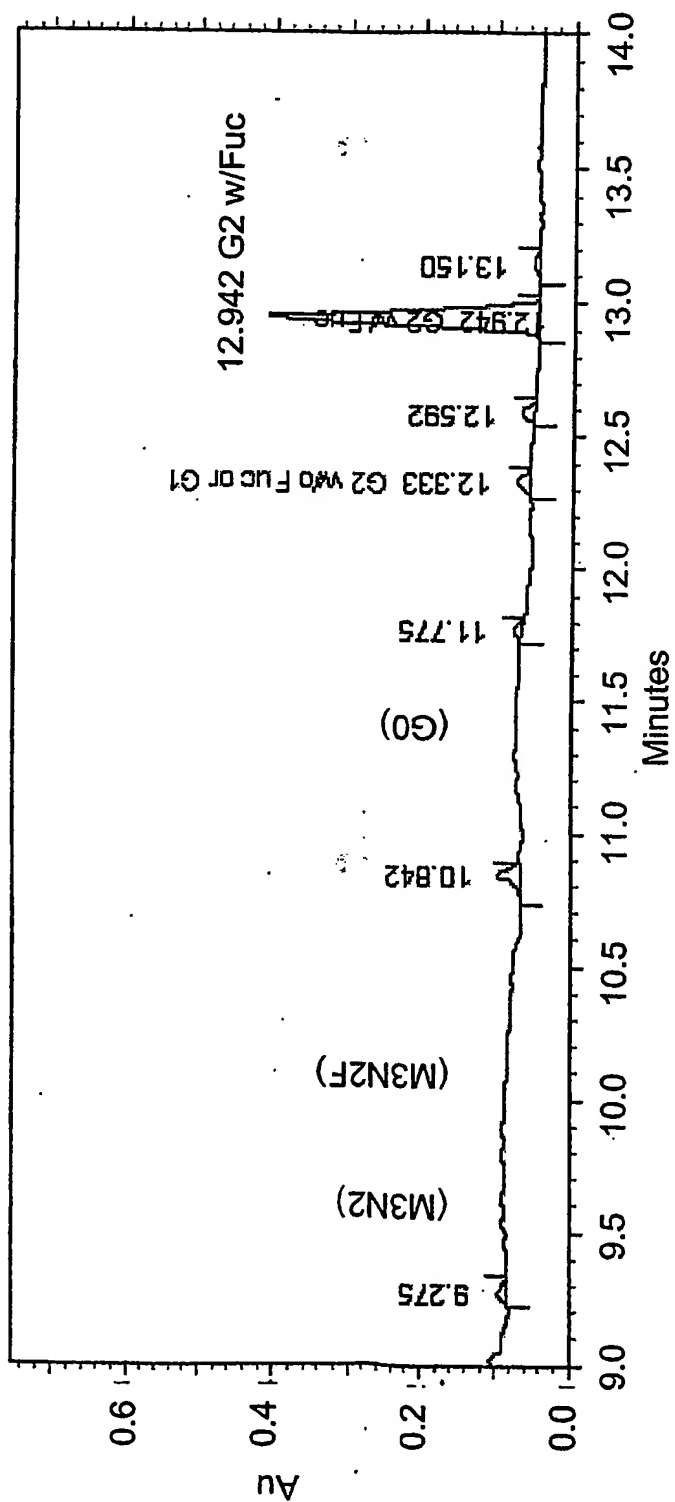


FIG. 103C

366/498

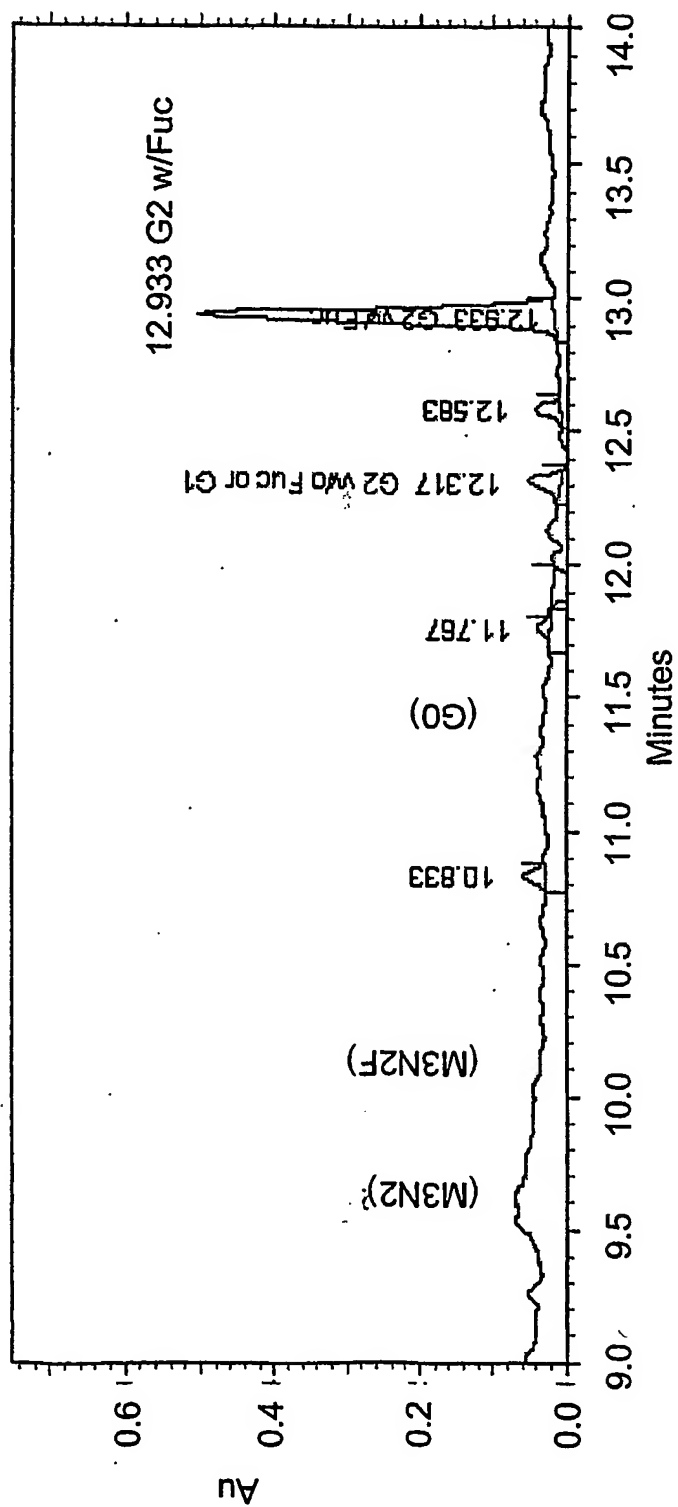


FIG. 103D

367/498

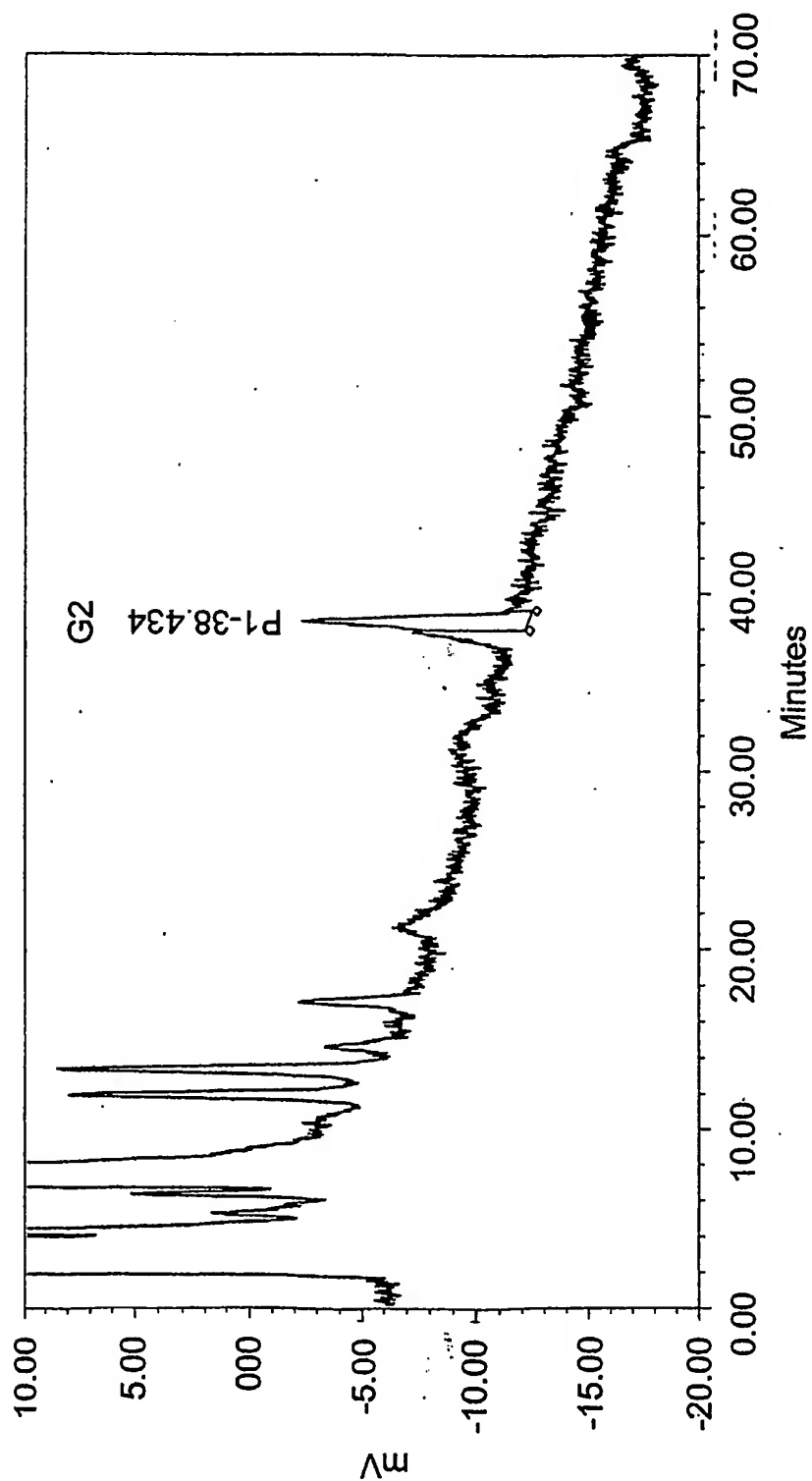


FIG. 104A

368/498

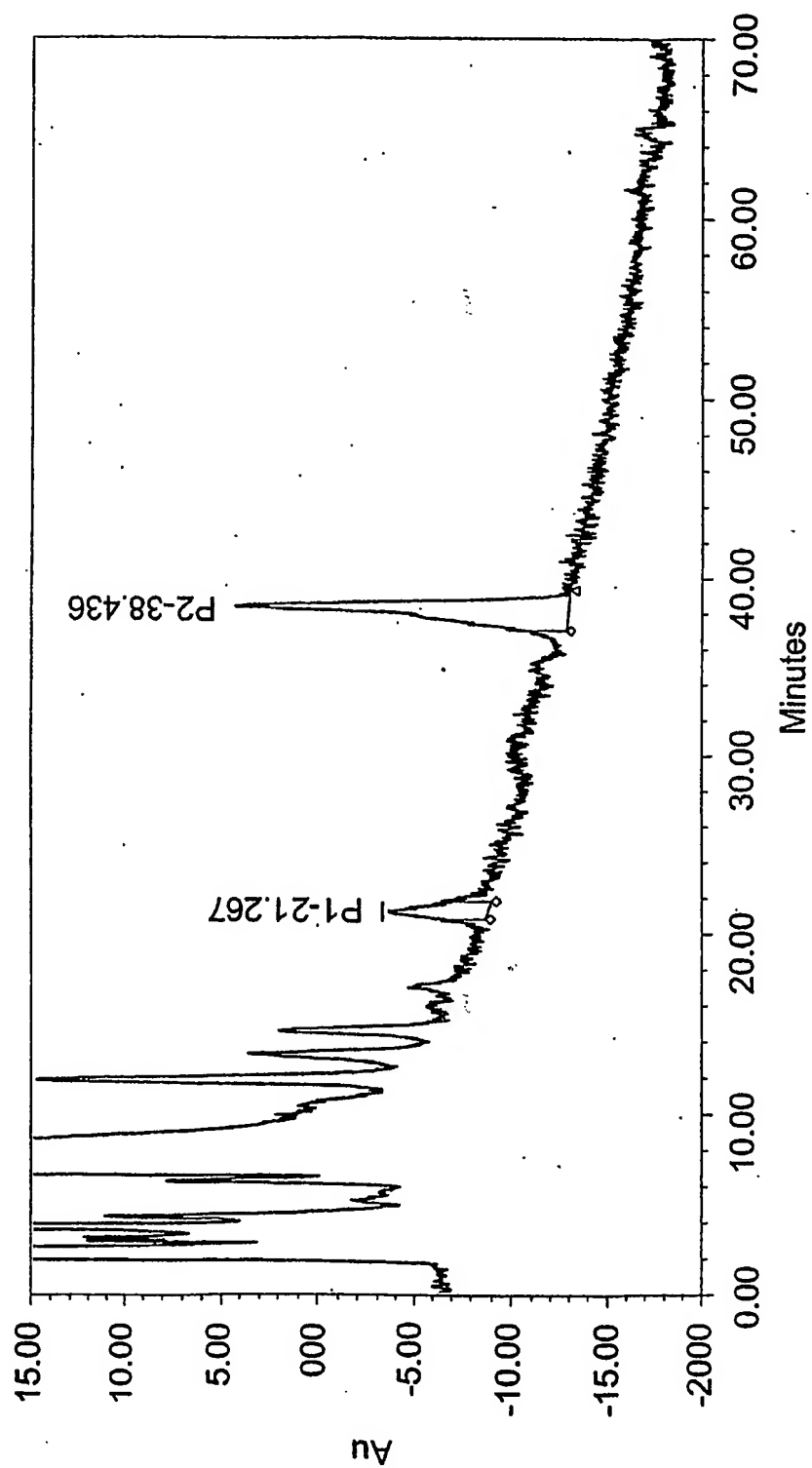


FIG. 104B

369/498

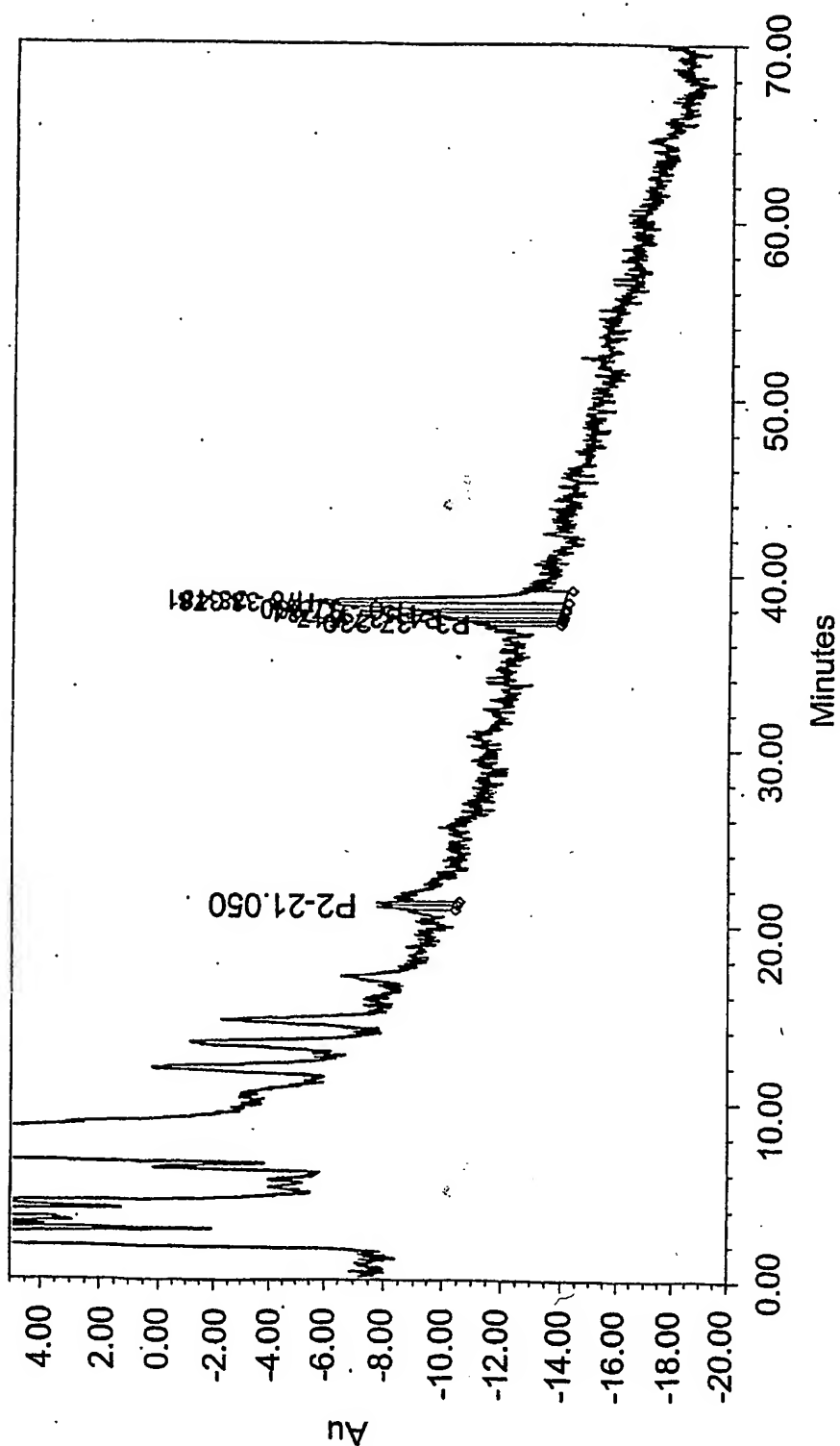


FIG. 104C



370/498

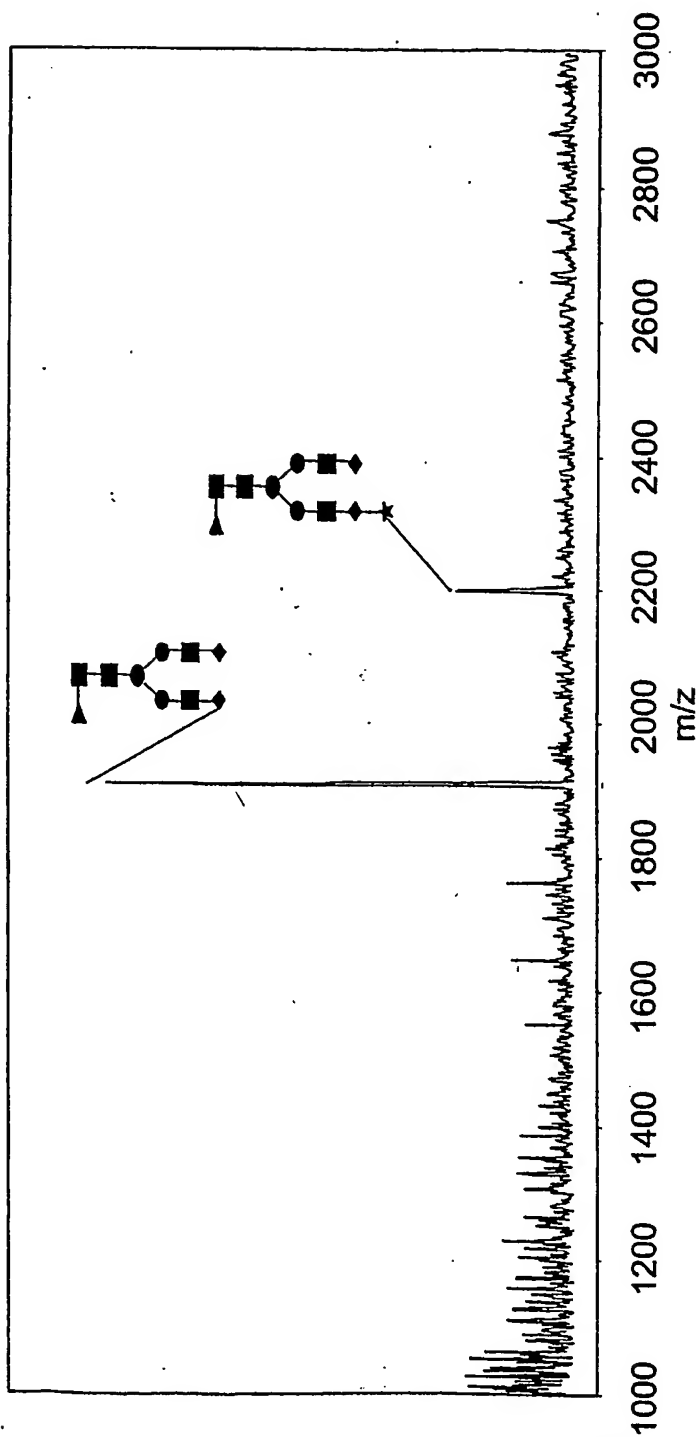


FIG. 105A

371/498

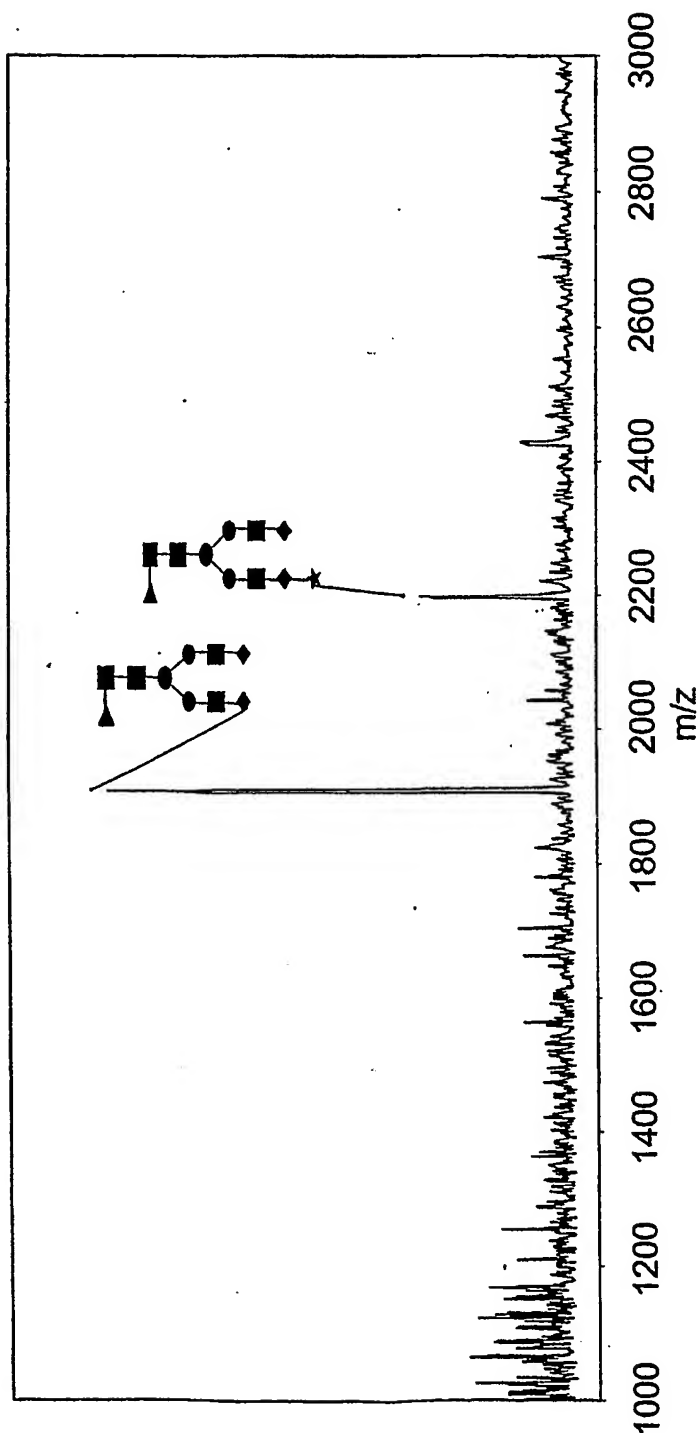


FIG. 105B

372/498

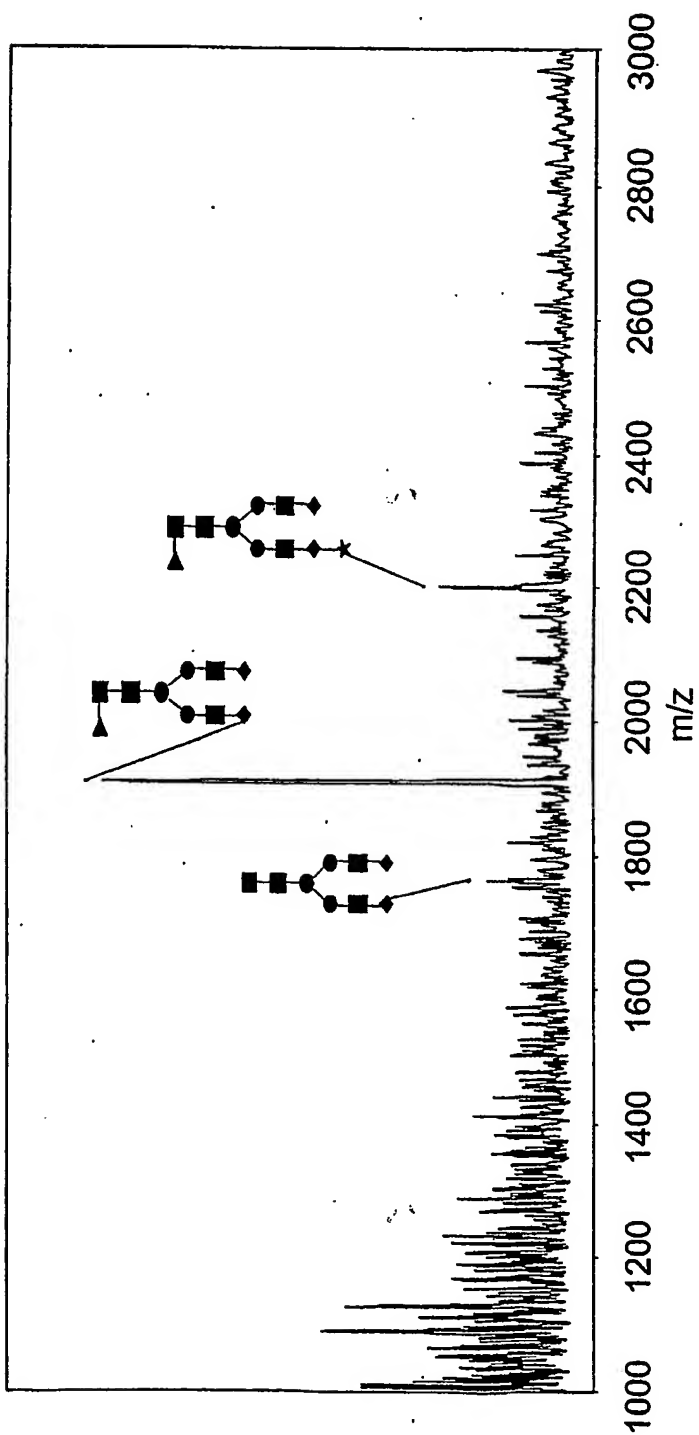


FIG. 105C

373/498

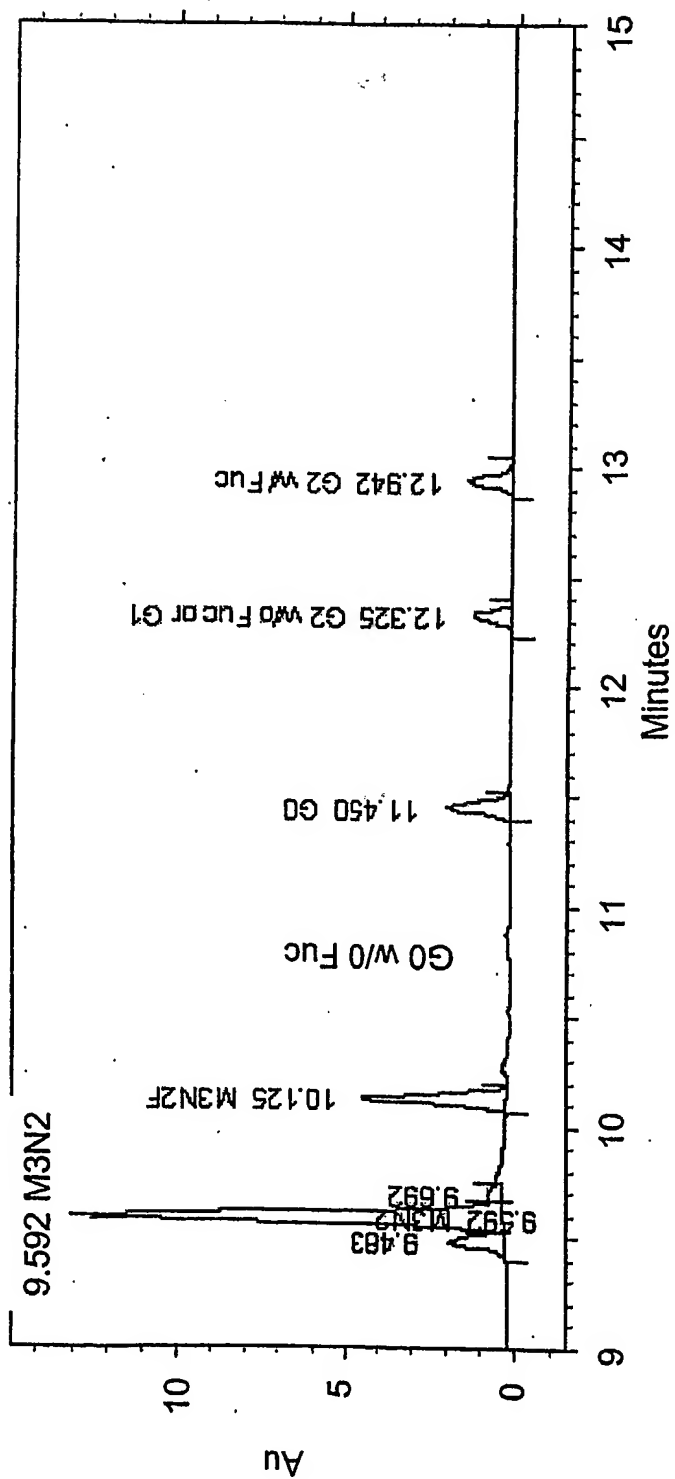


FIG. 106A

374/498

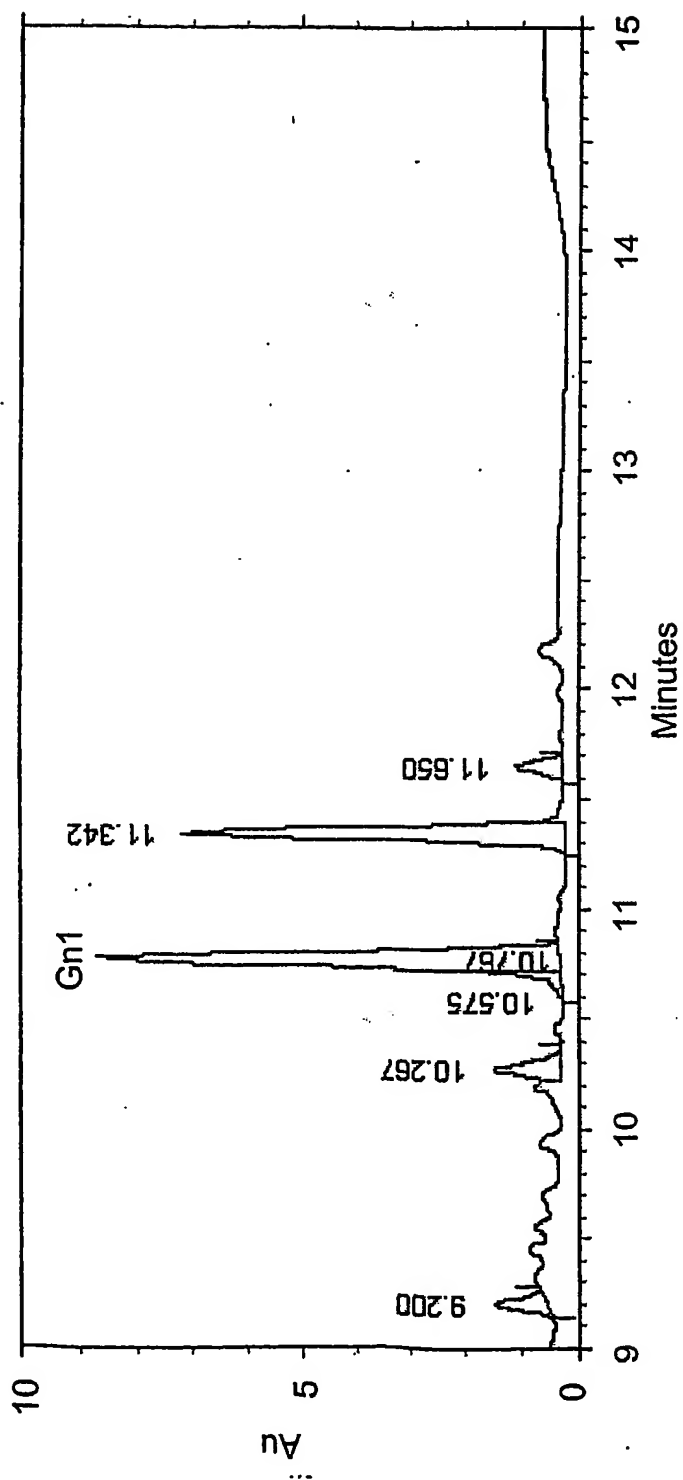


FIG. 106B

375/498

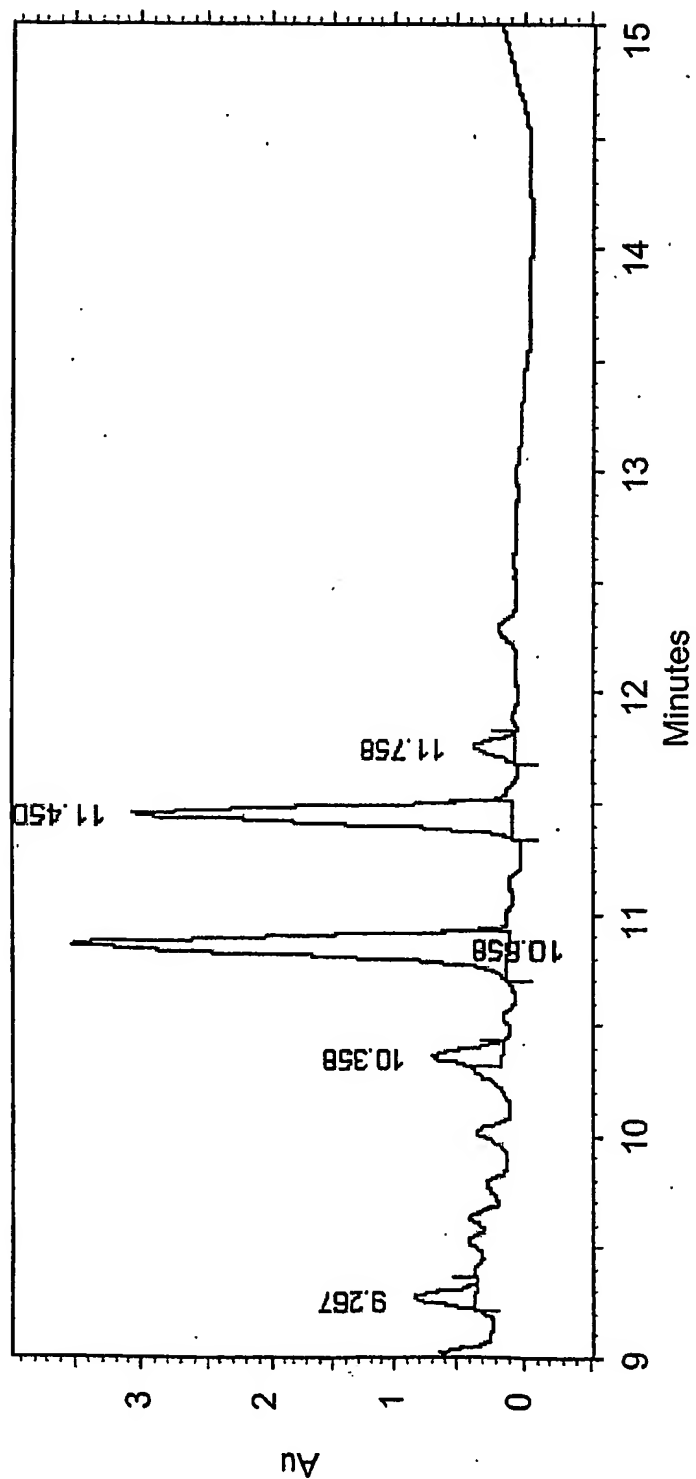


FIG. 106C

376/498

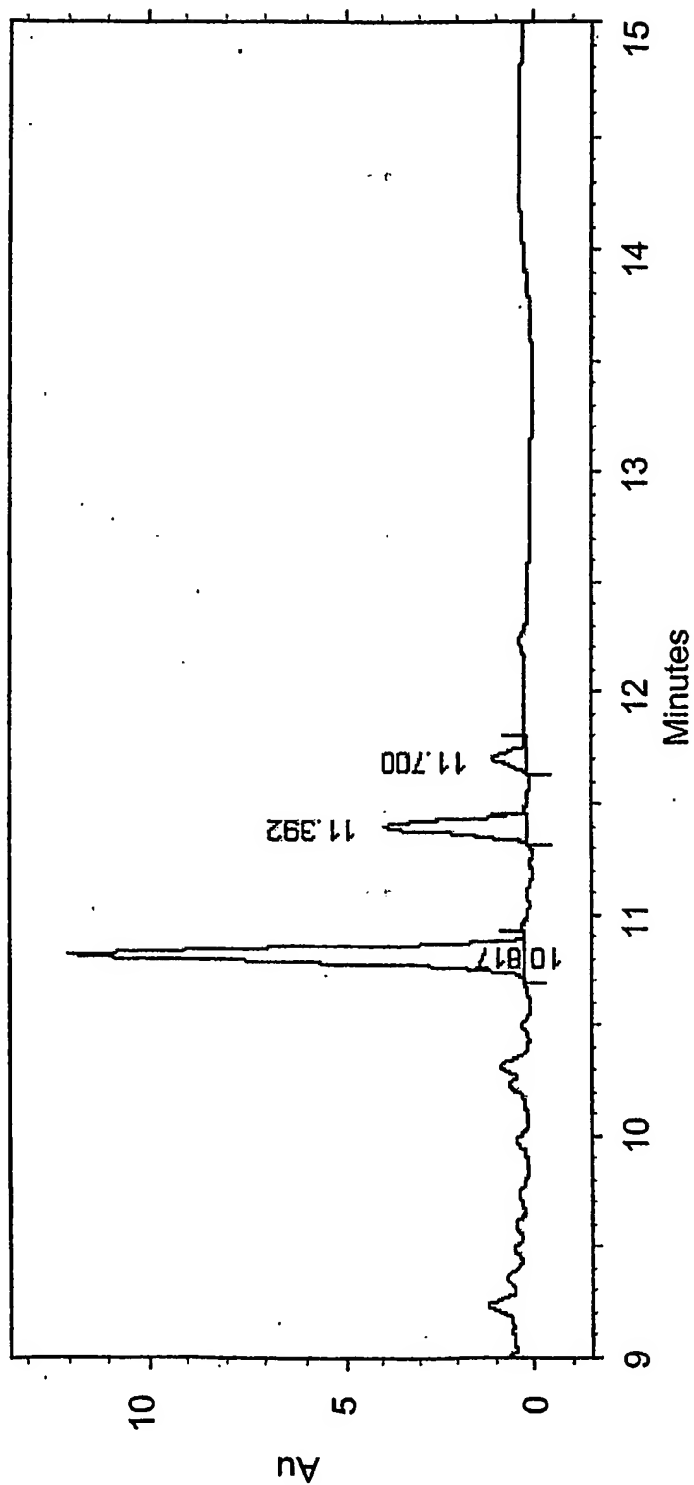


FIG. 106D

377/498

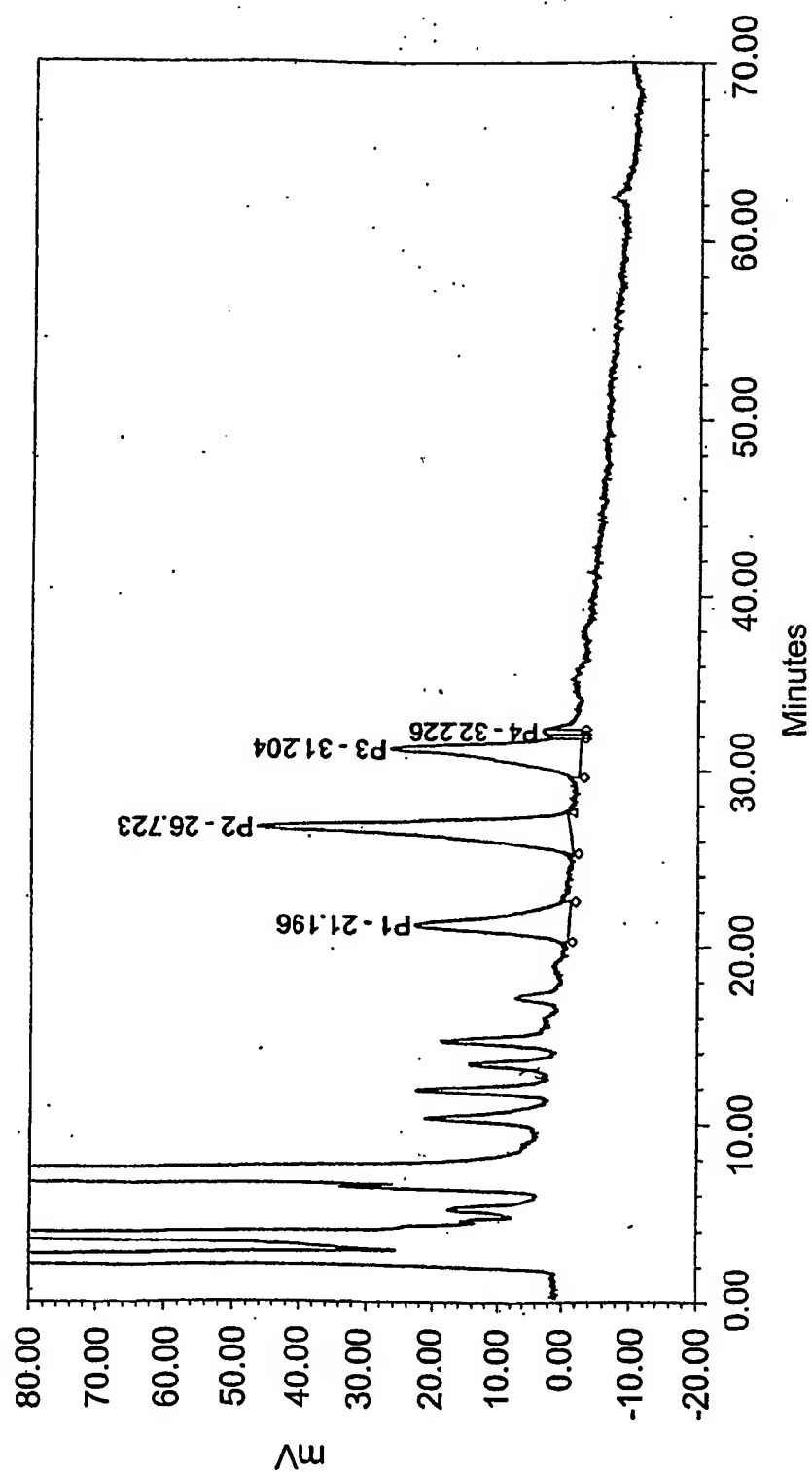


FIG. 107A



378/498

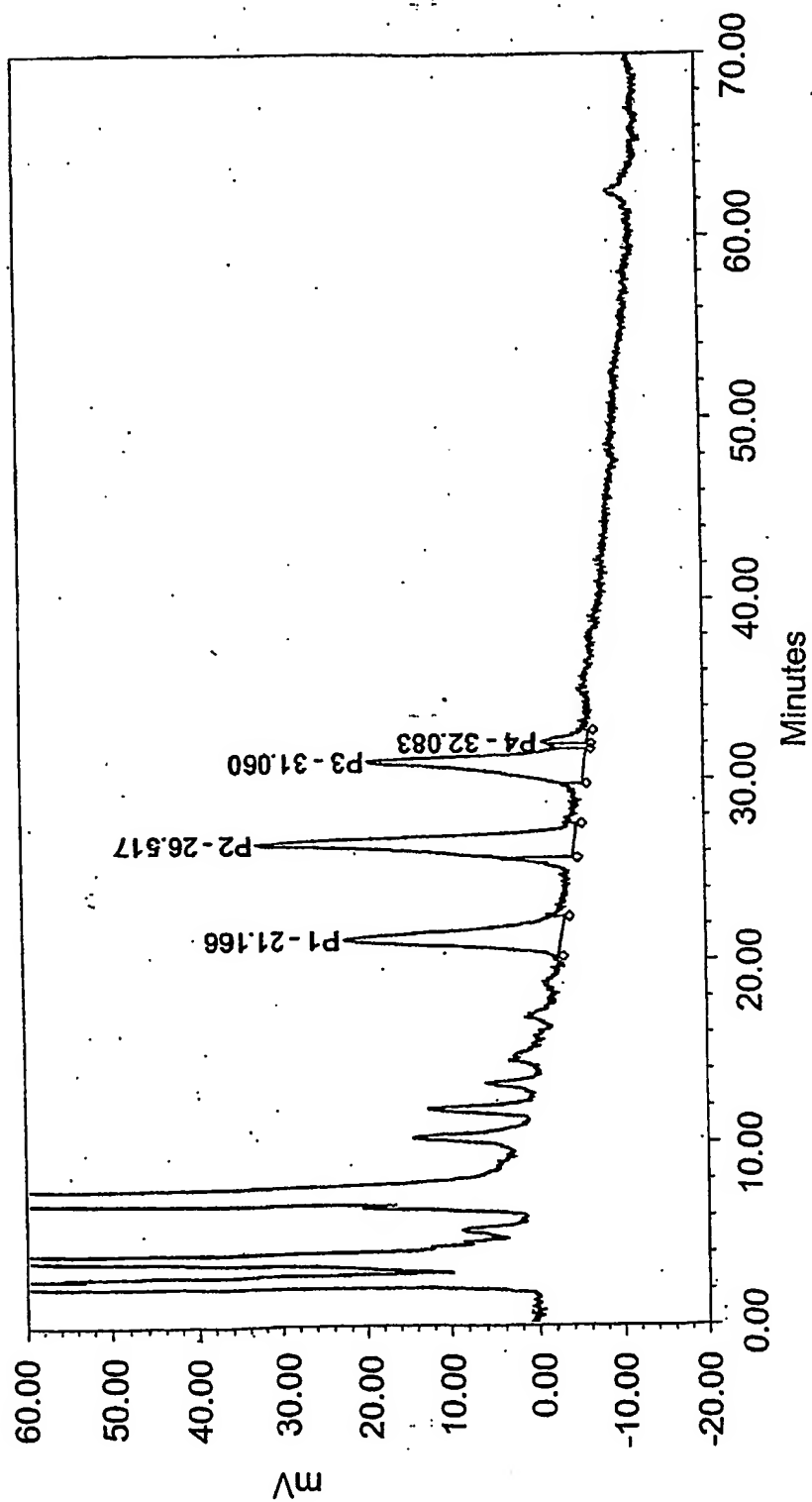


FIG. 107B

379/498

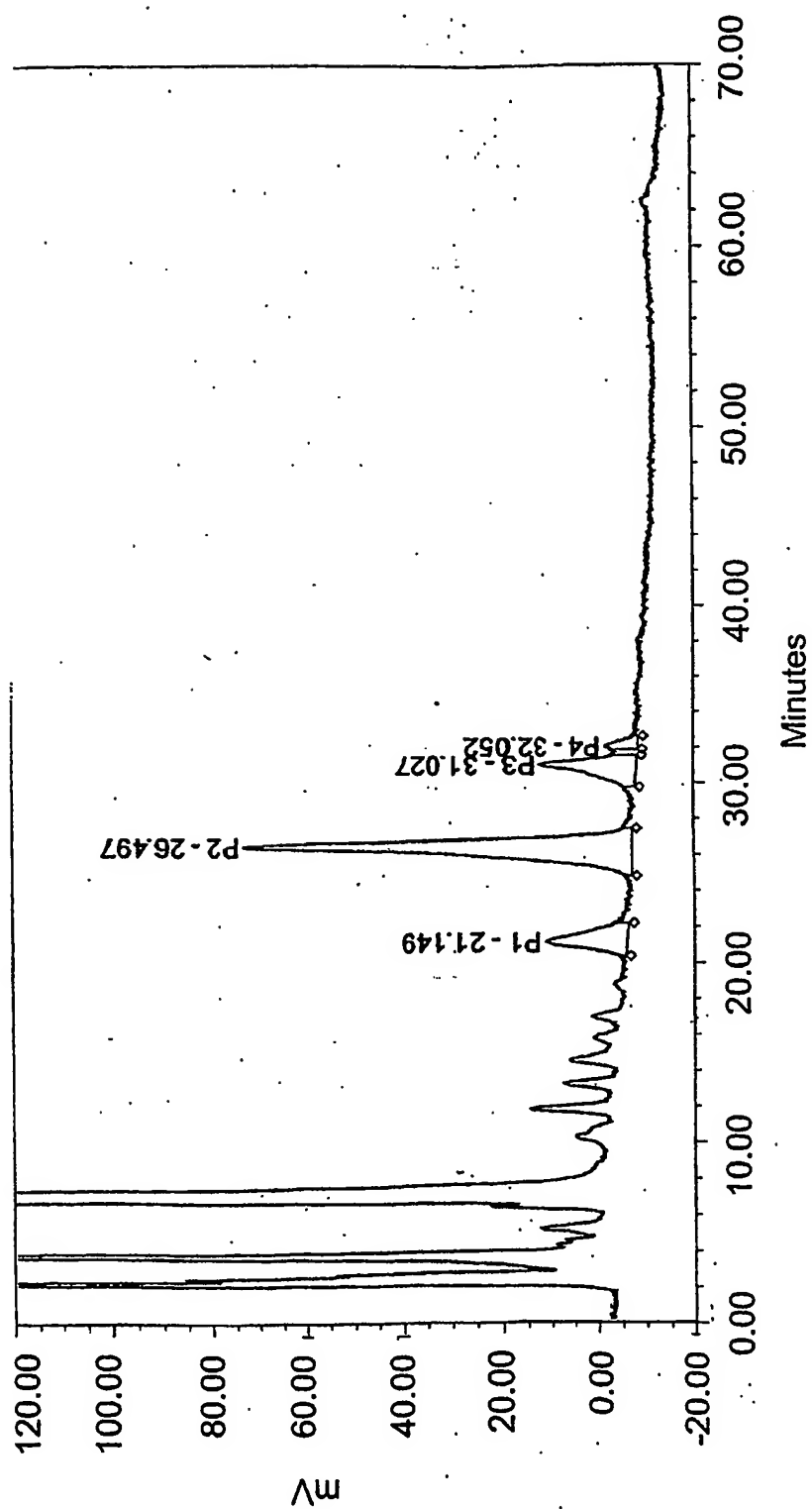


FIG. 107C

380/498

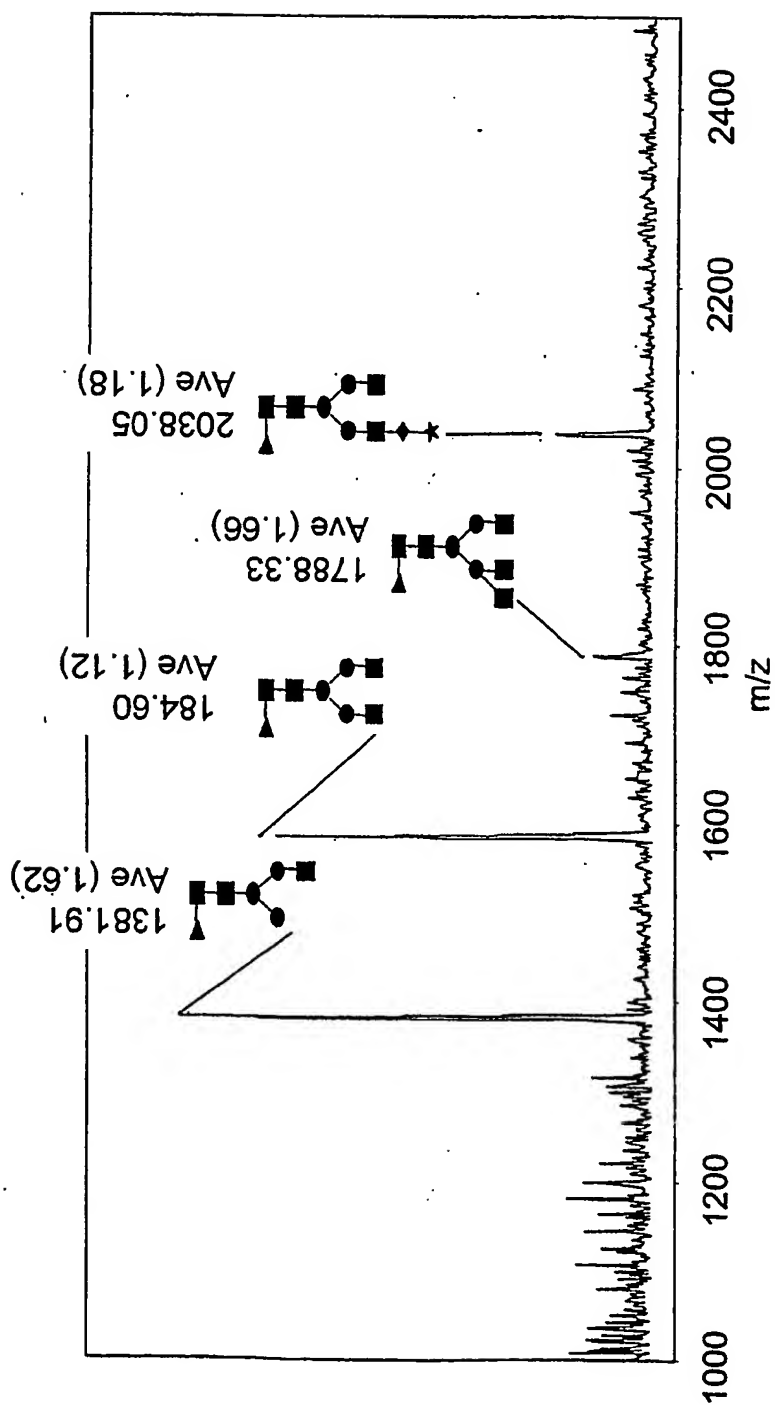


FIG. 108A

381/498

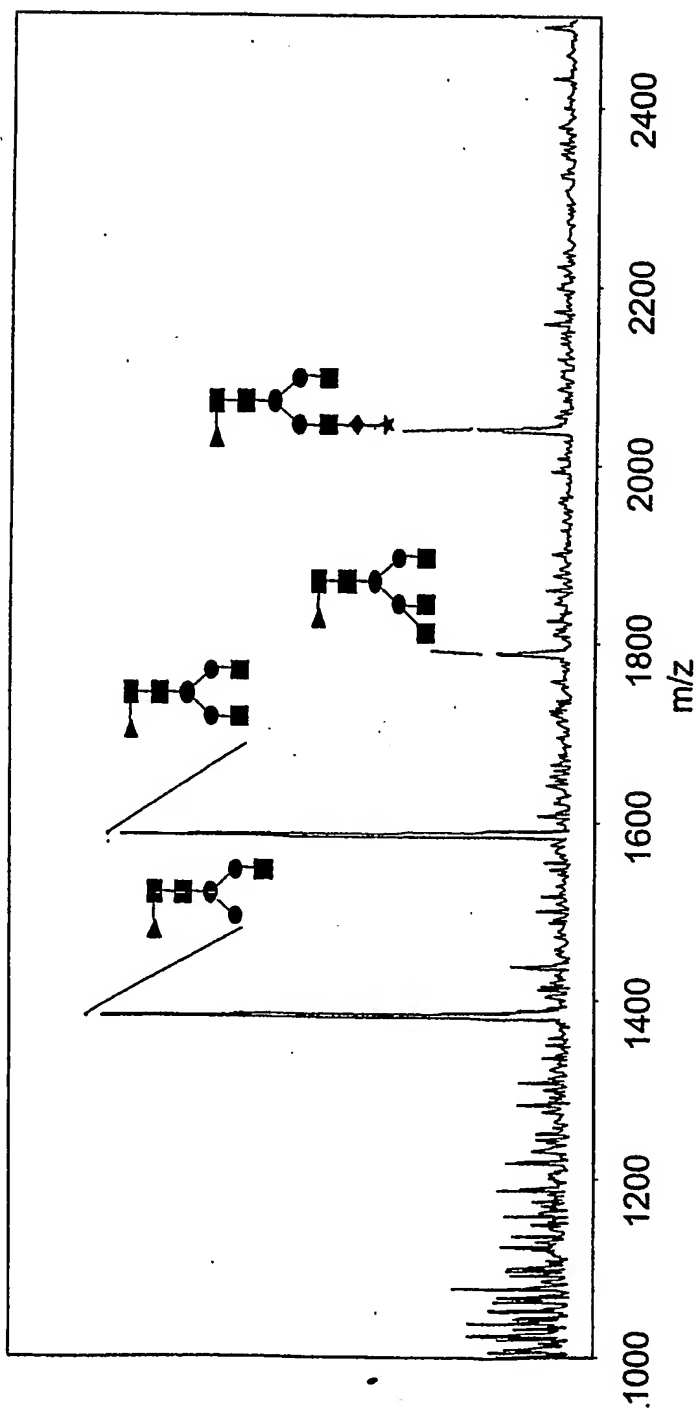


FIG. 108B

382/498

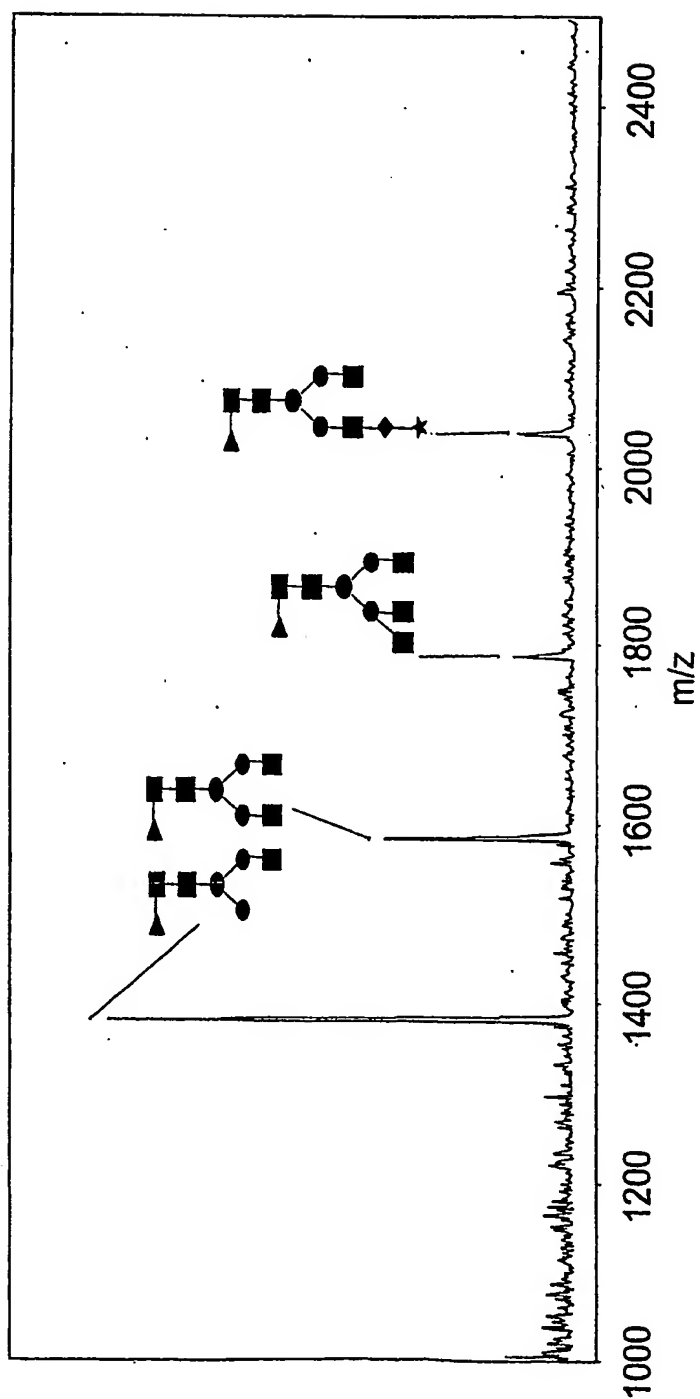


FIG. 108C

383/498

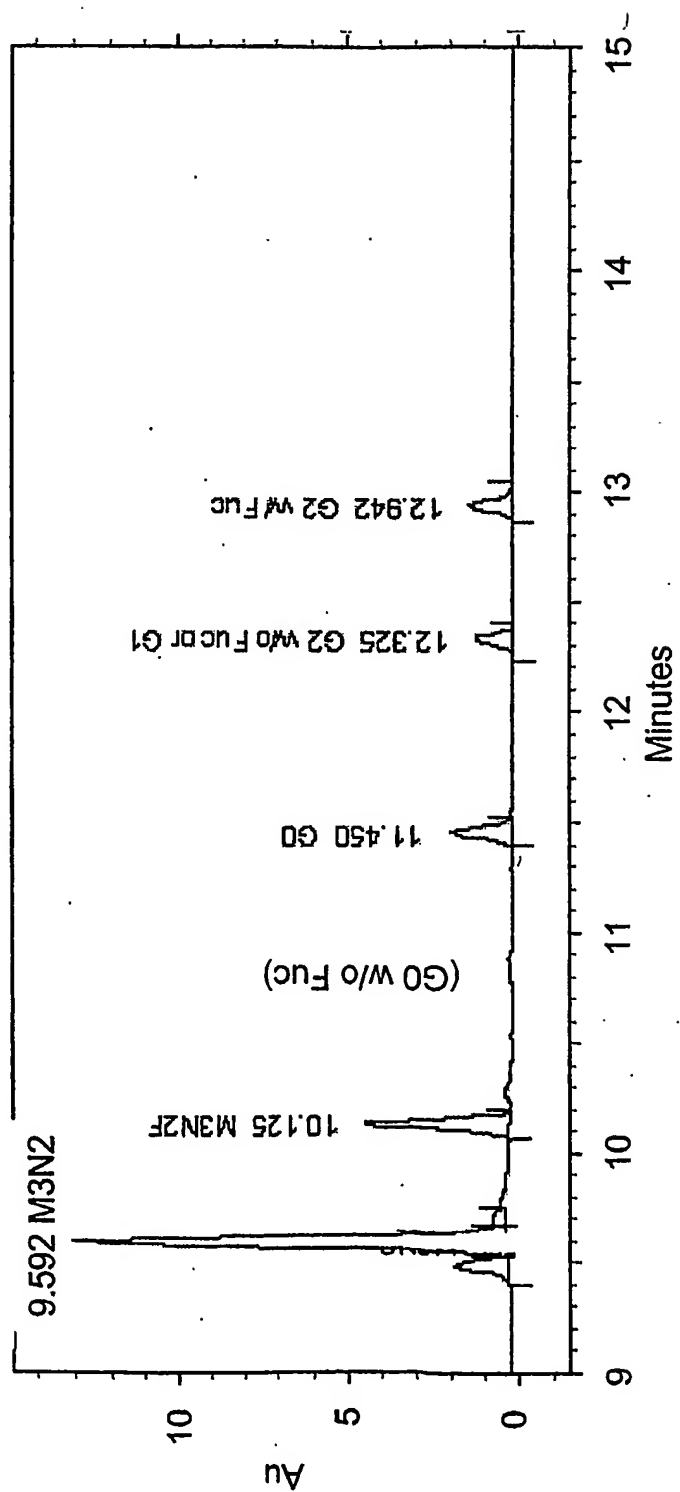


FIG. 109A

384/498

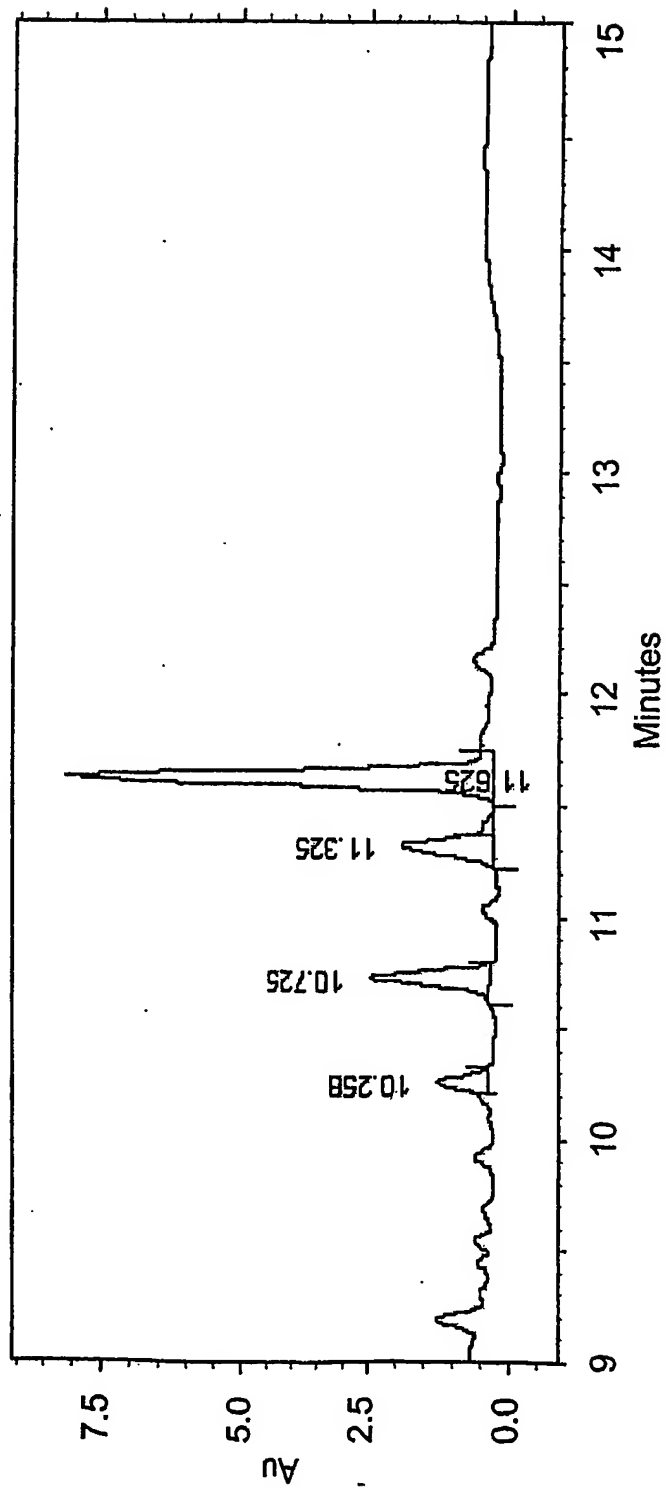


FIG. 109B

385/498

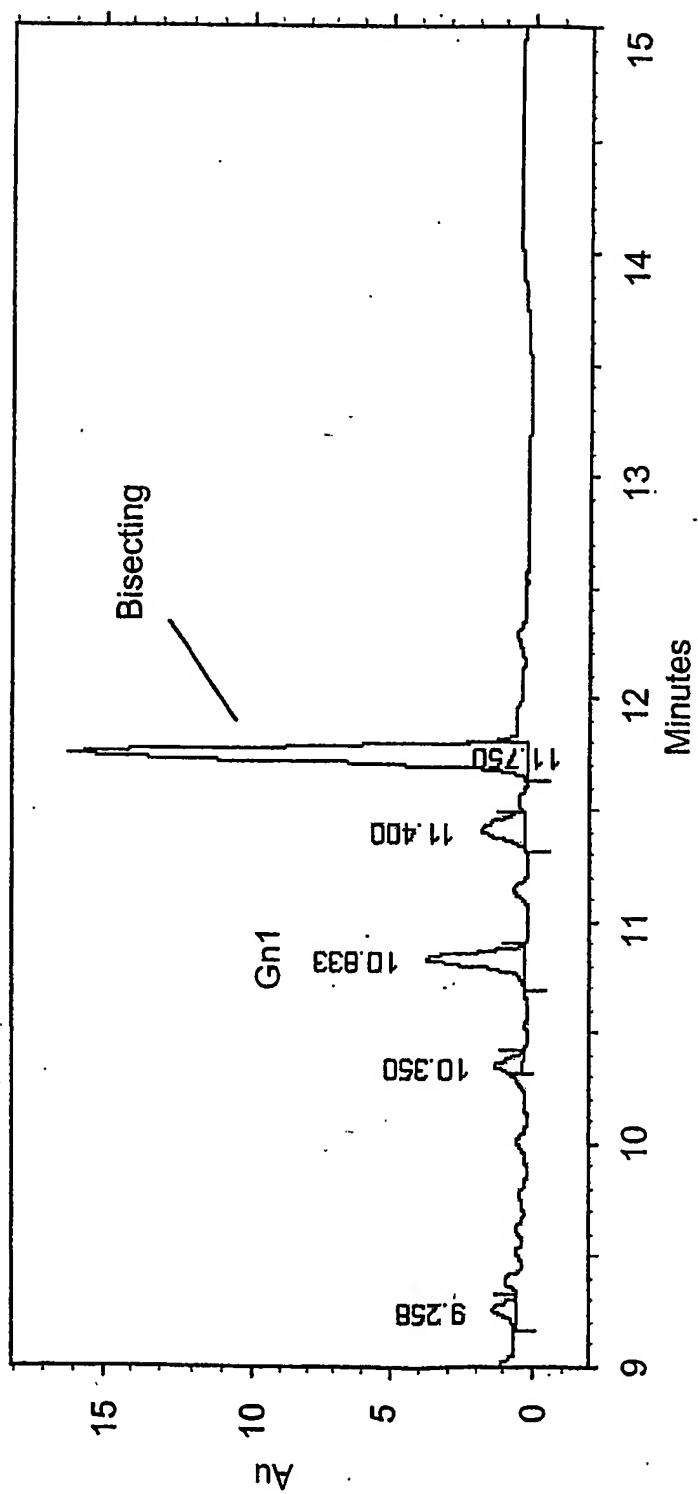


FIG. 109C



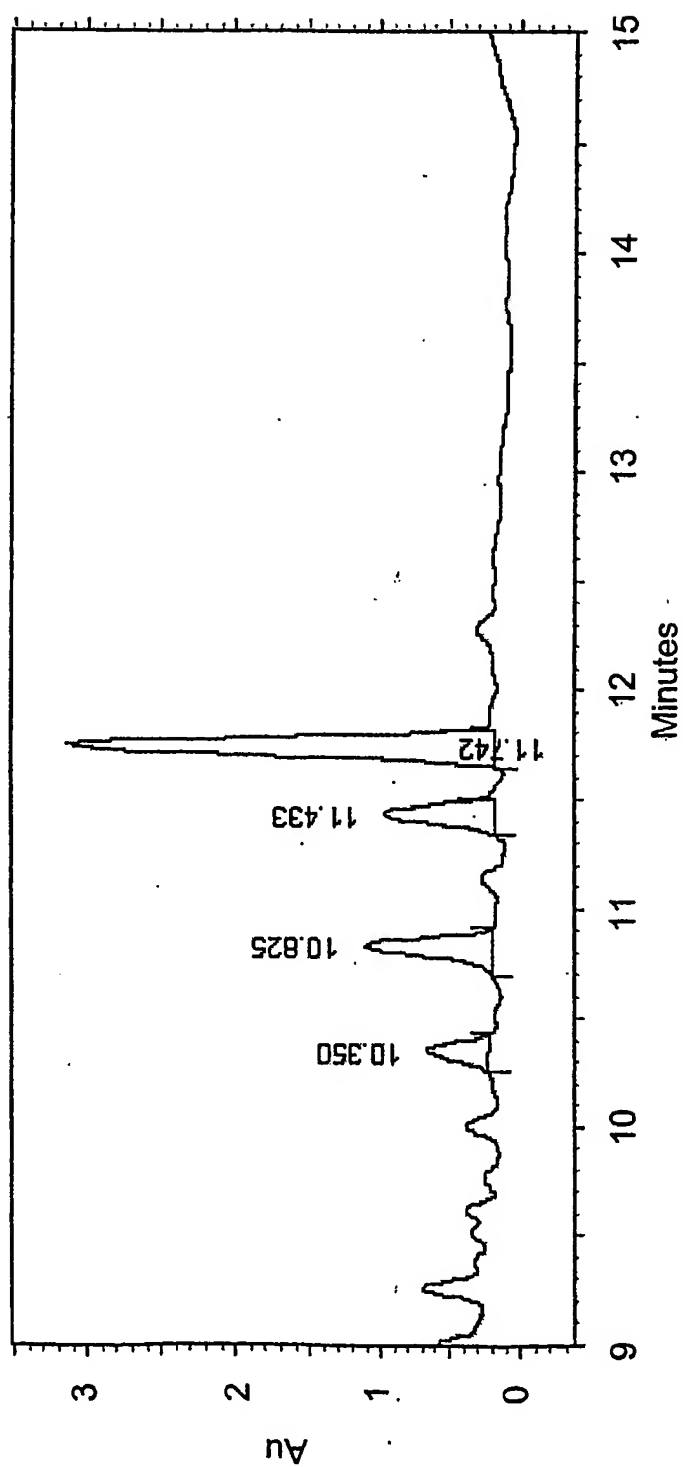


FIG. 109D

387/498

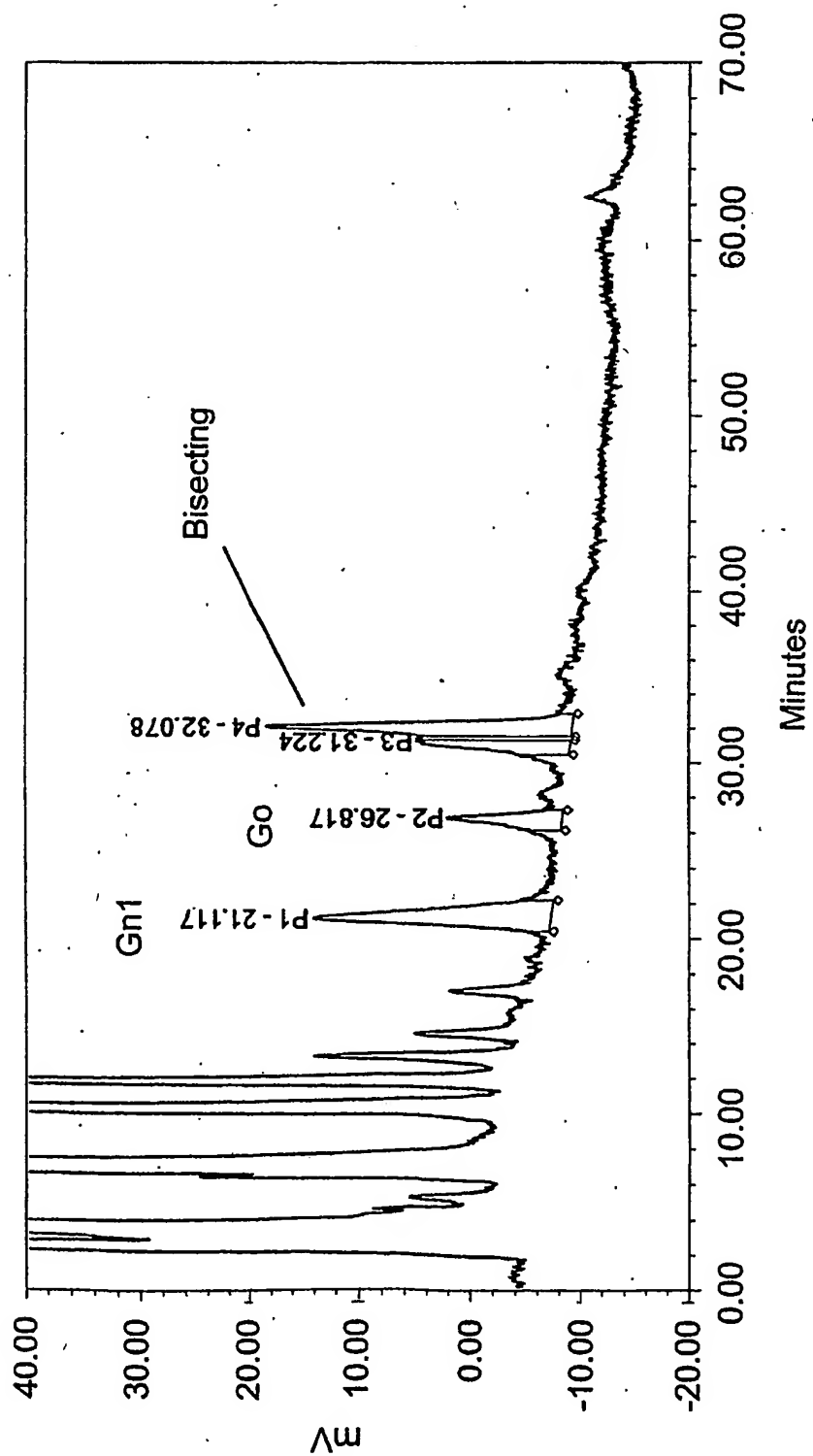


FIG. 110A

388/498

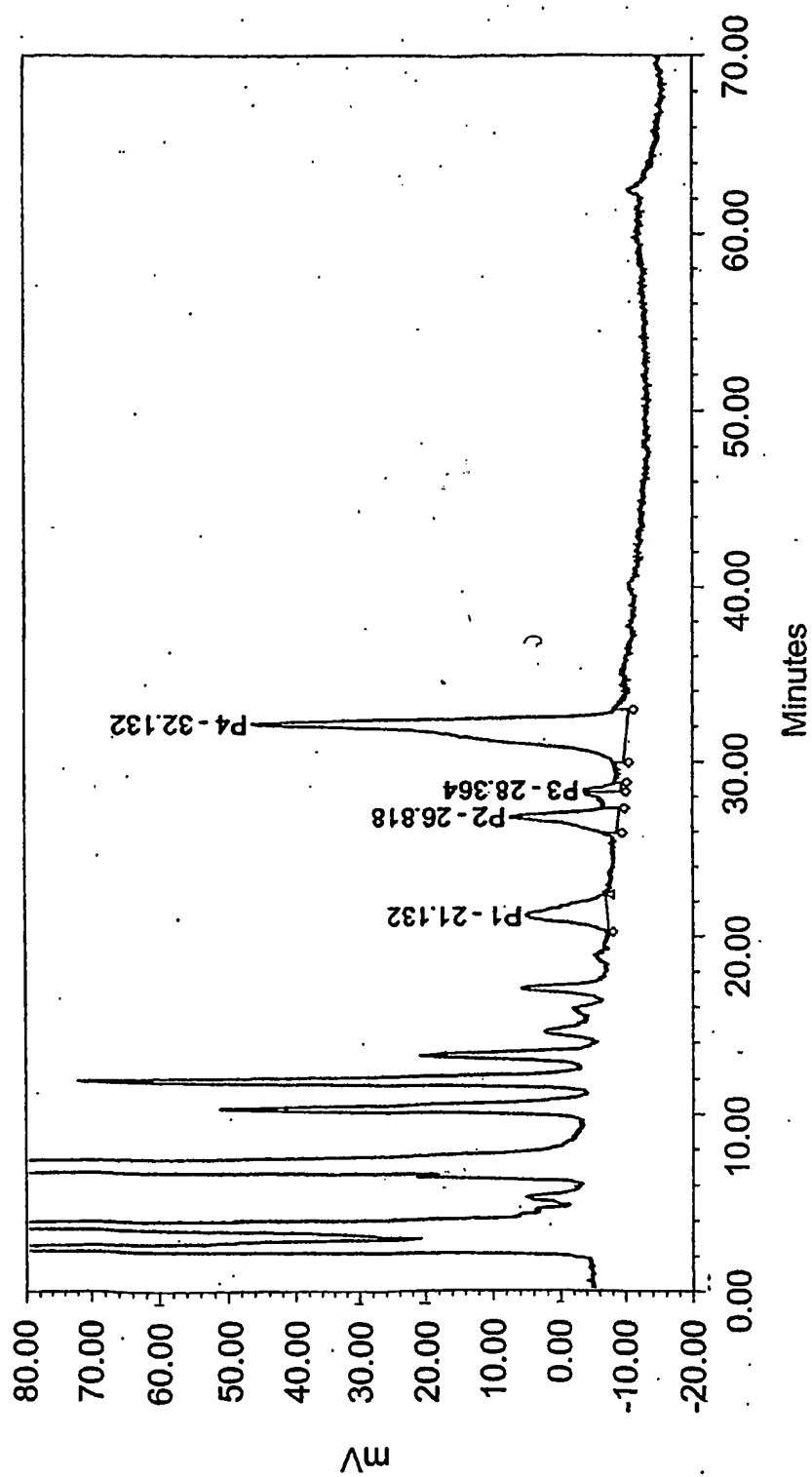


FIG. 110B

389/498

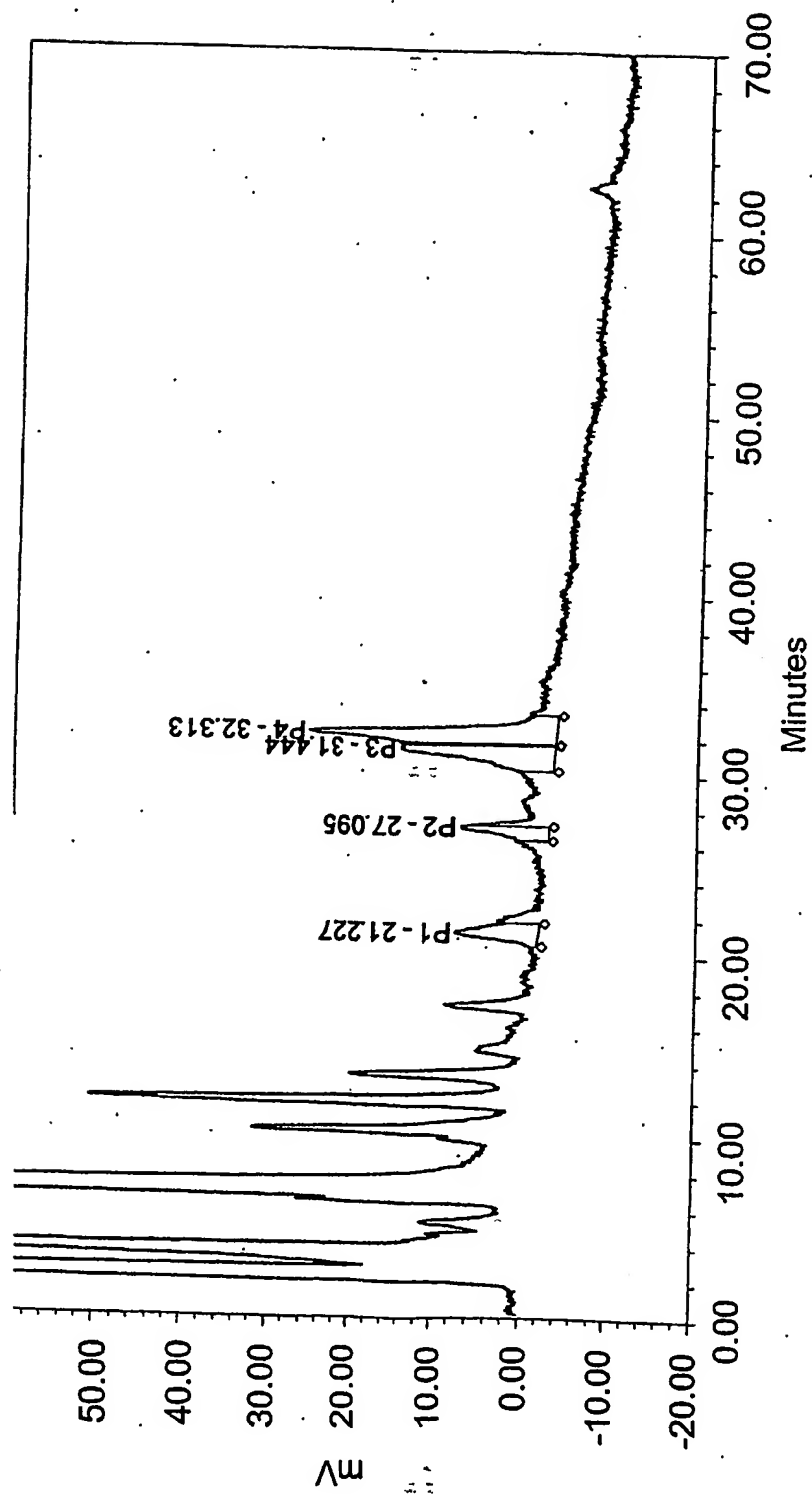


FIG. 110C

390/498

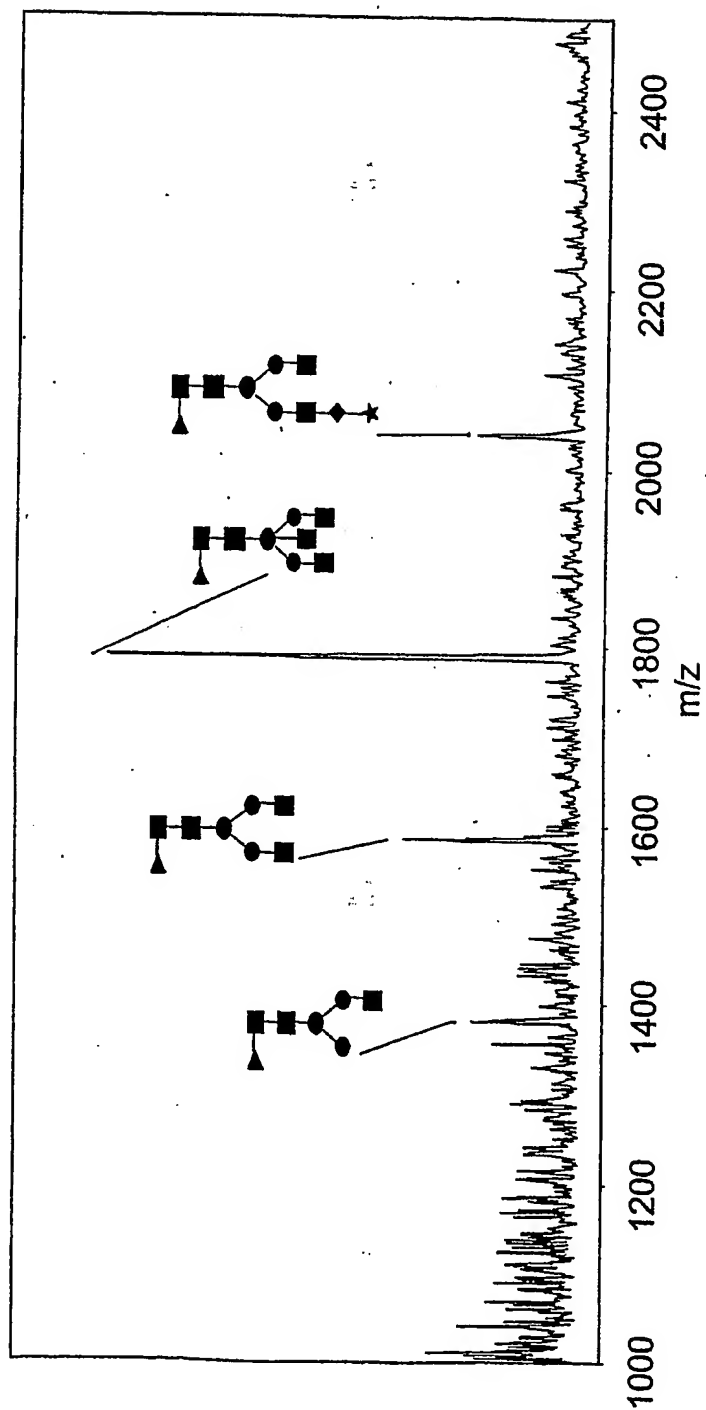


FIG. 111A

391/498

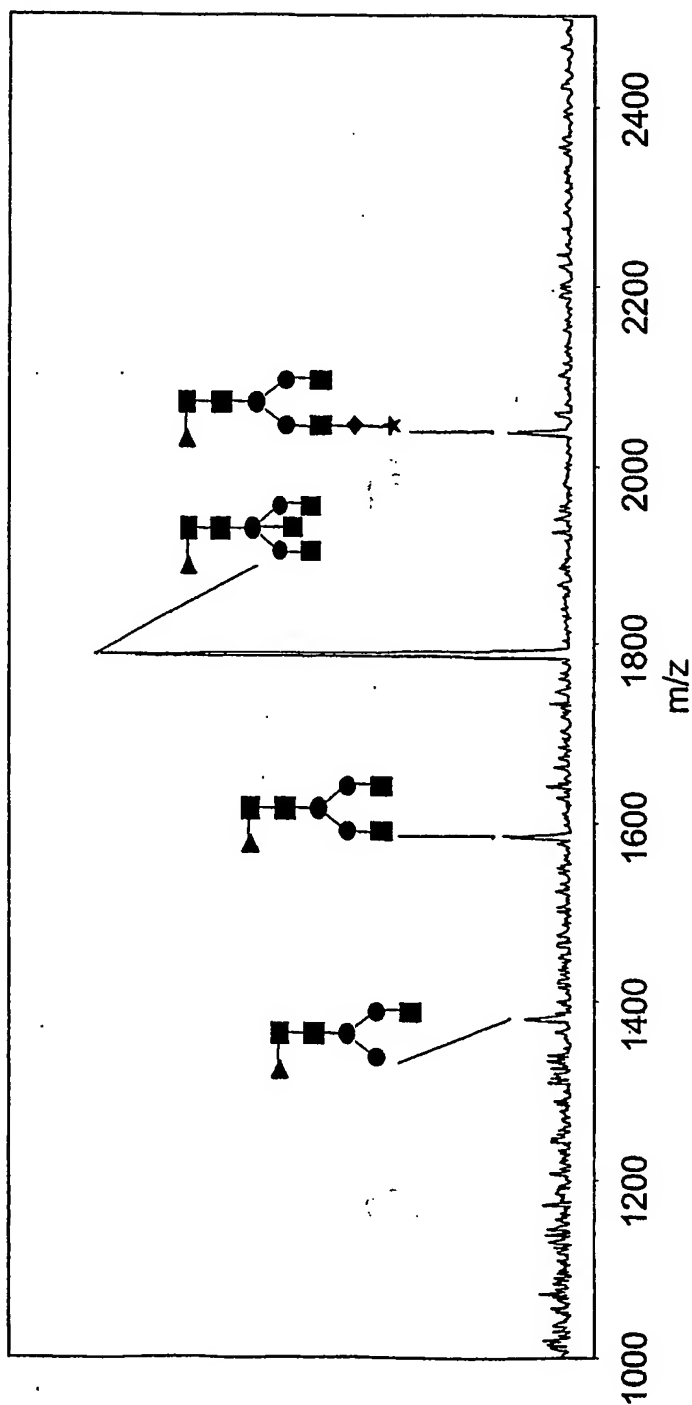


FIG. 111B

392/498

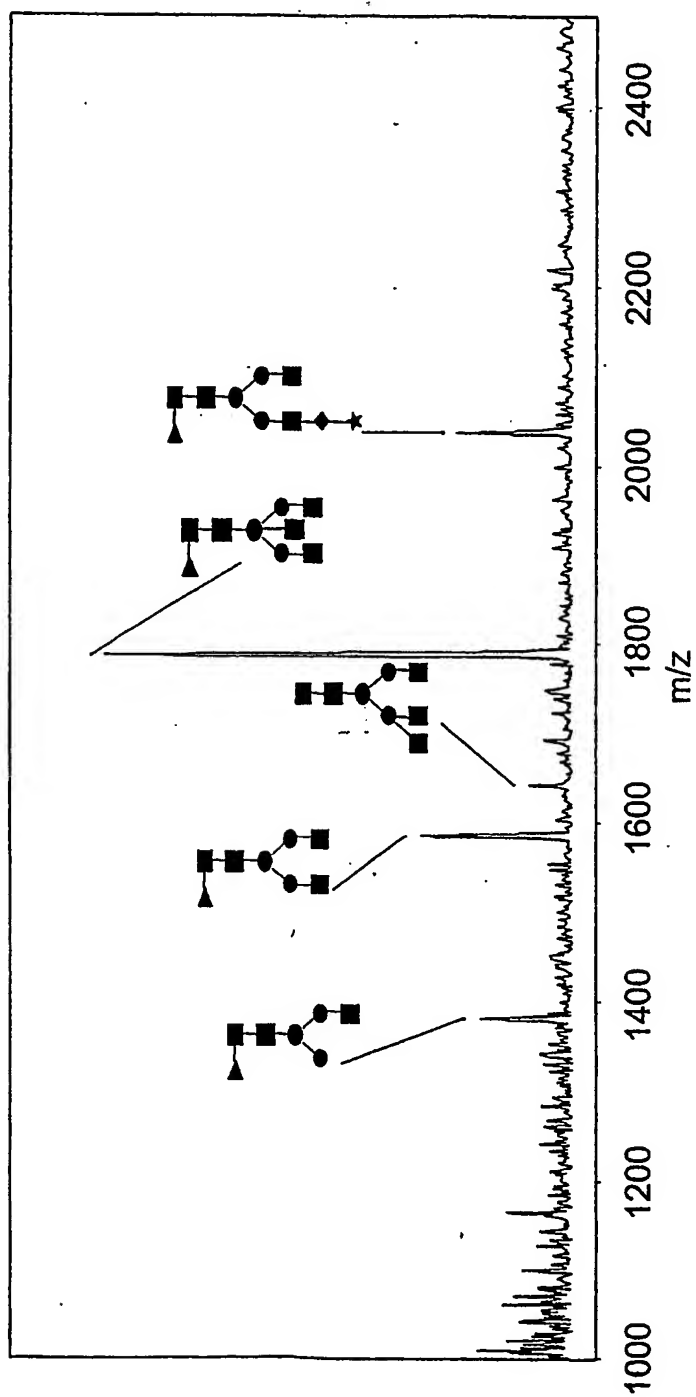


FIG. 111C

393/498

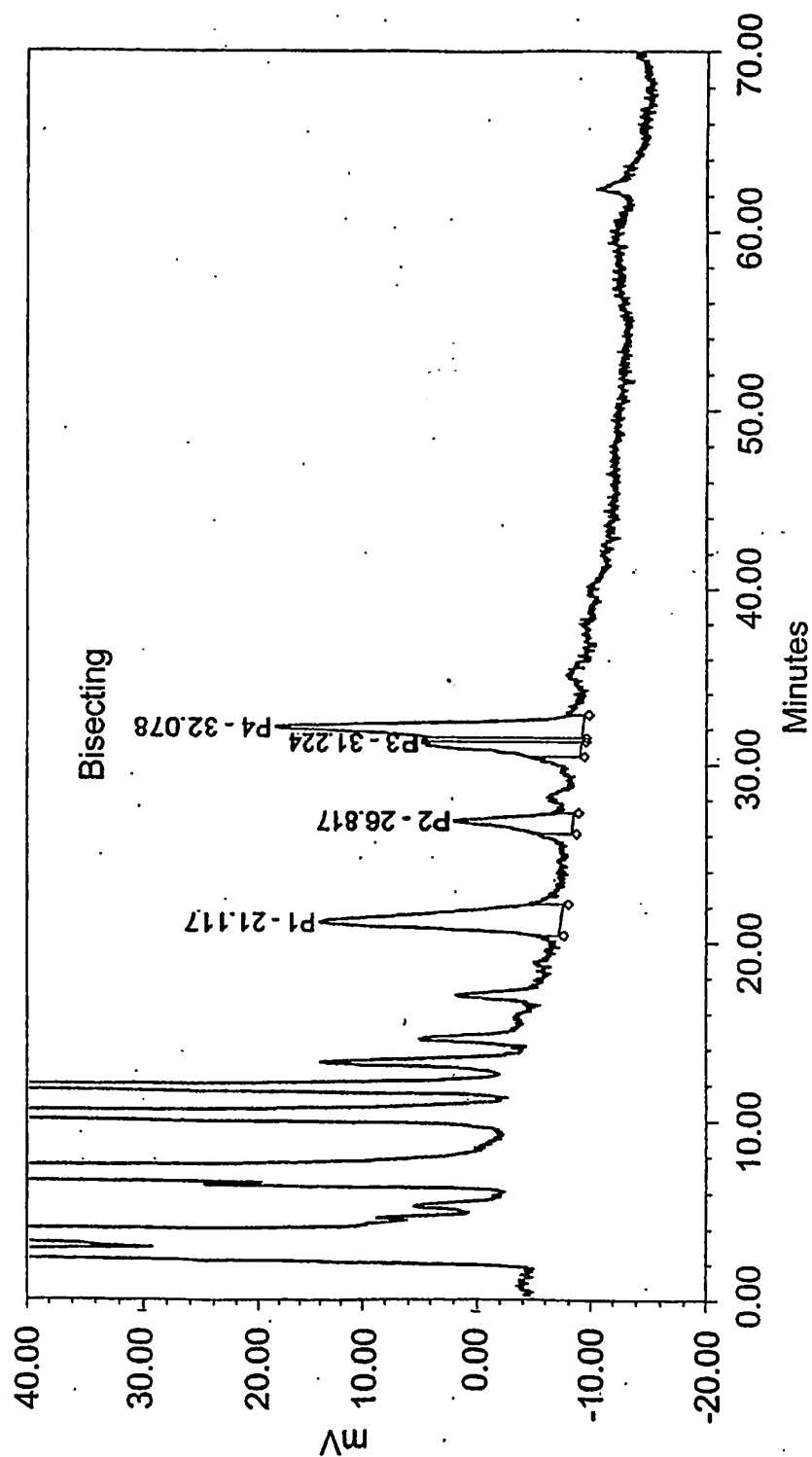


FIG. 112A



394/498

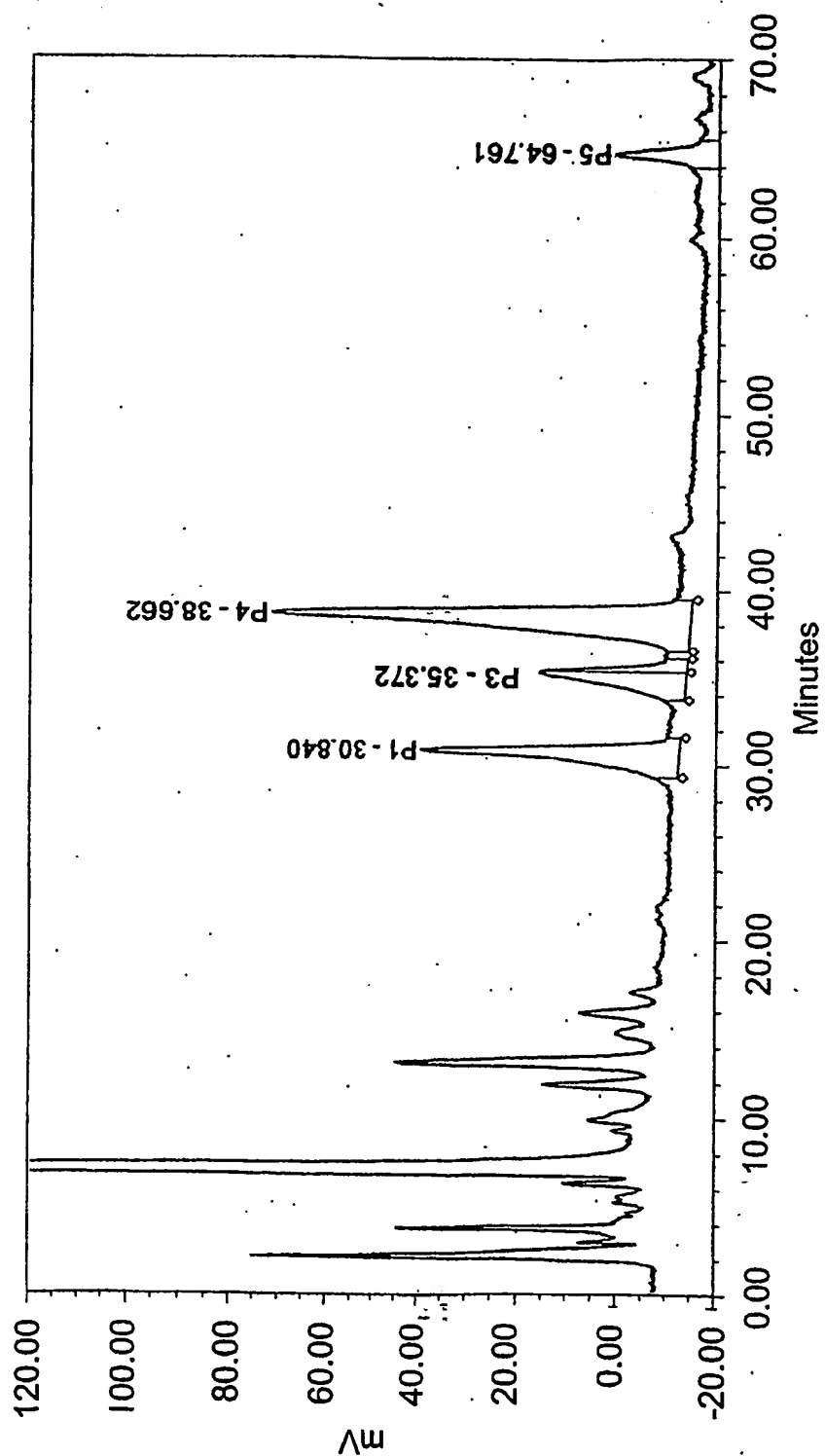


FIG. 112B

395/498

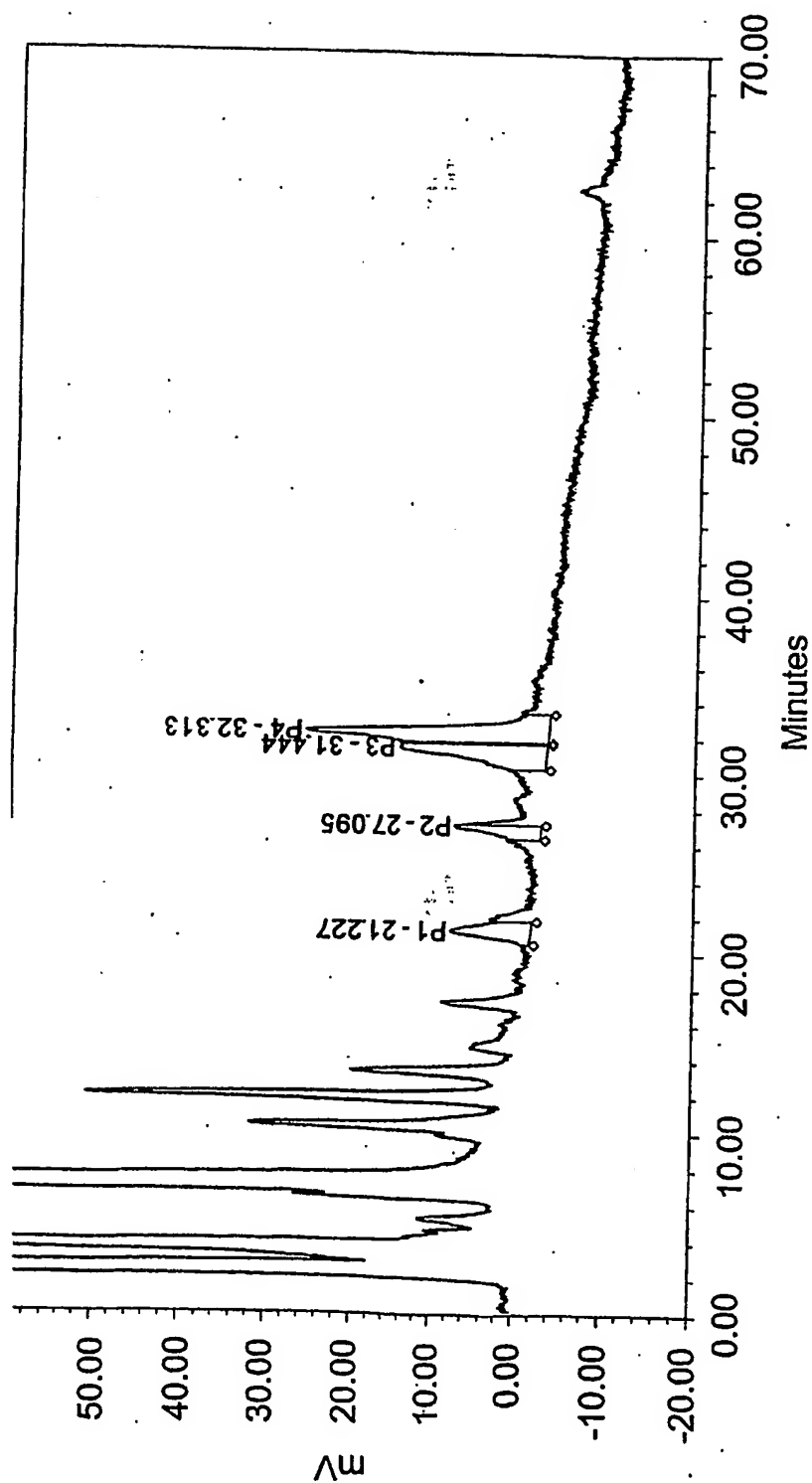


FIG. 112C

396/498

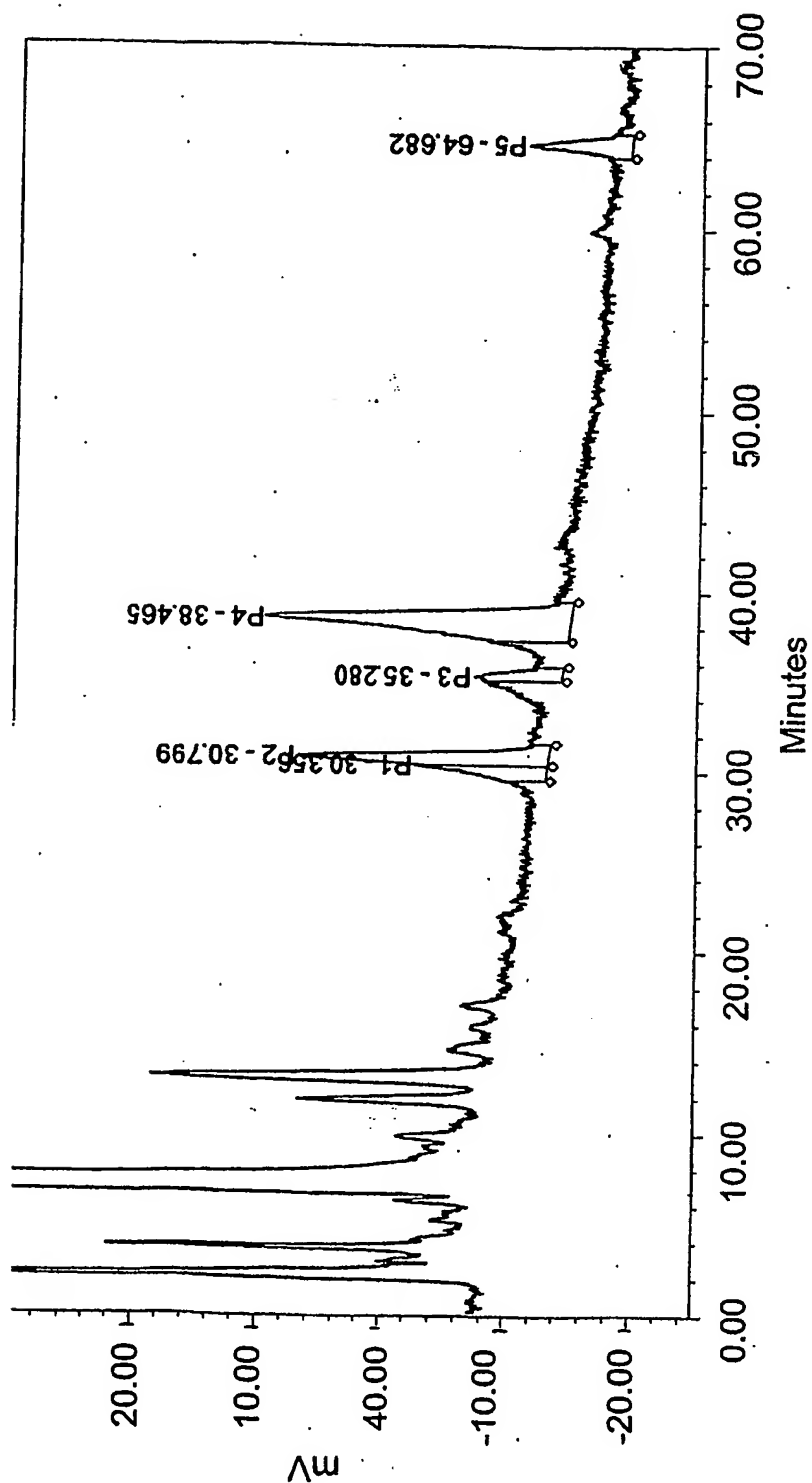


FIG. 112D

397/498

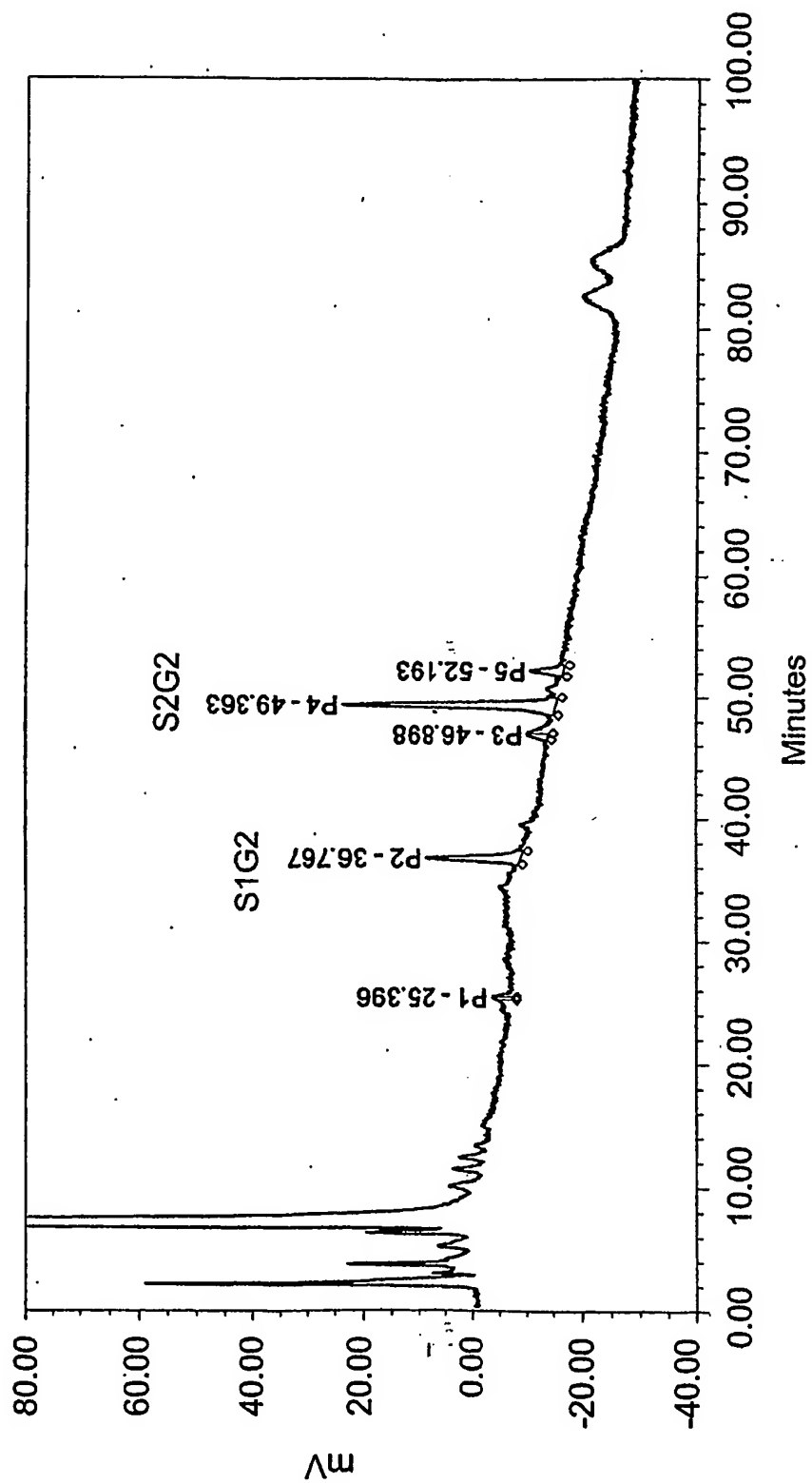


FIG. 113A

398/498

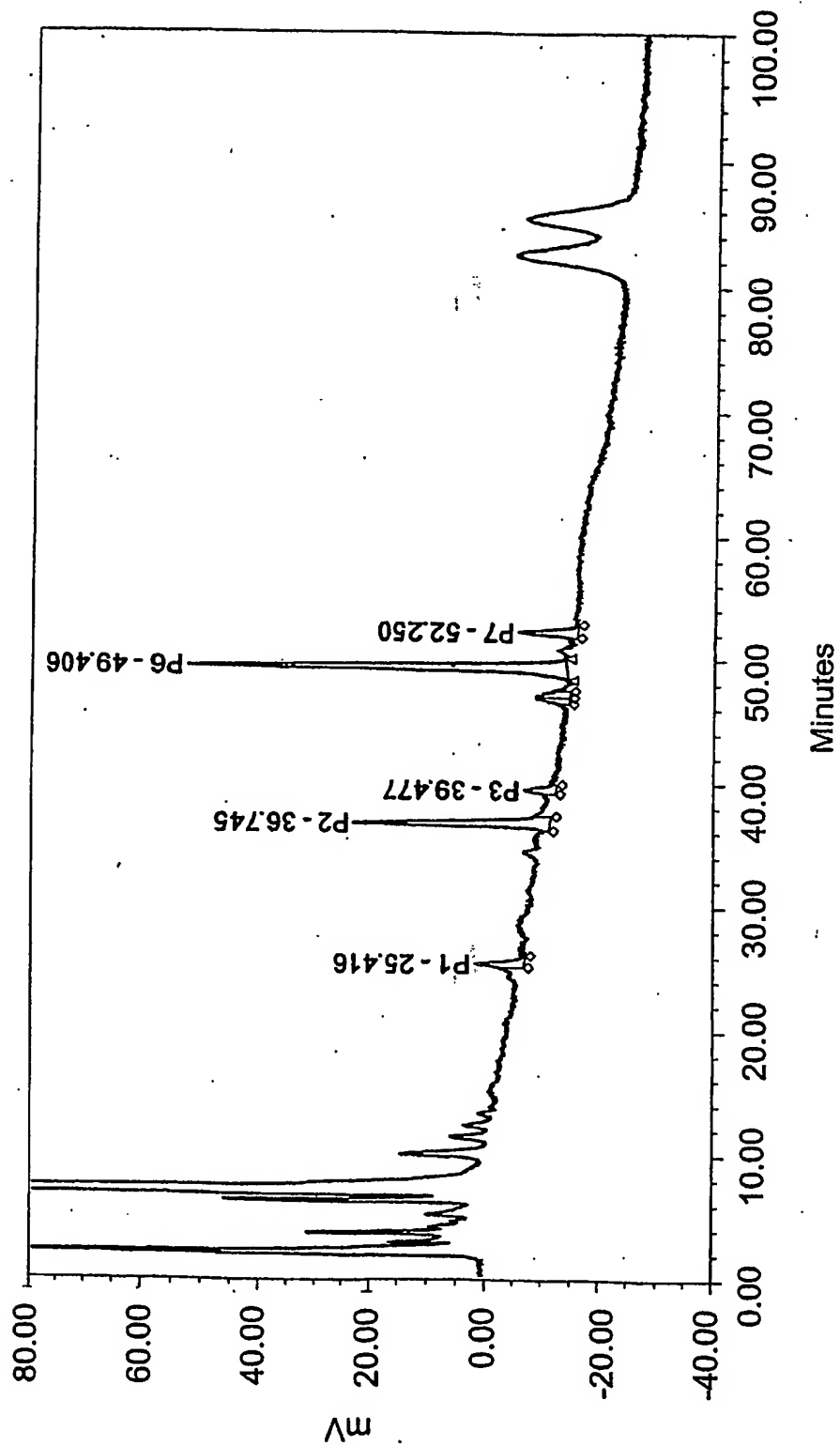


FIG. 113B

399/498

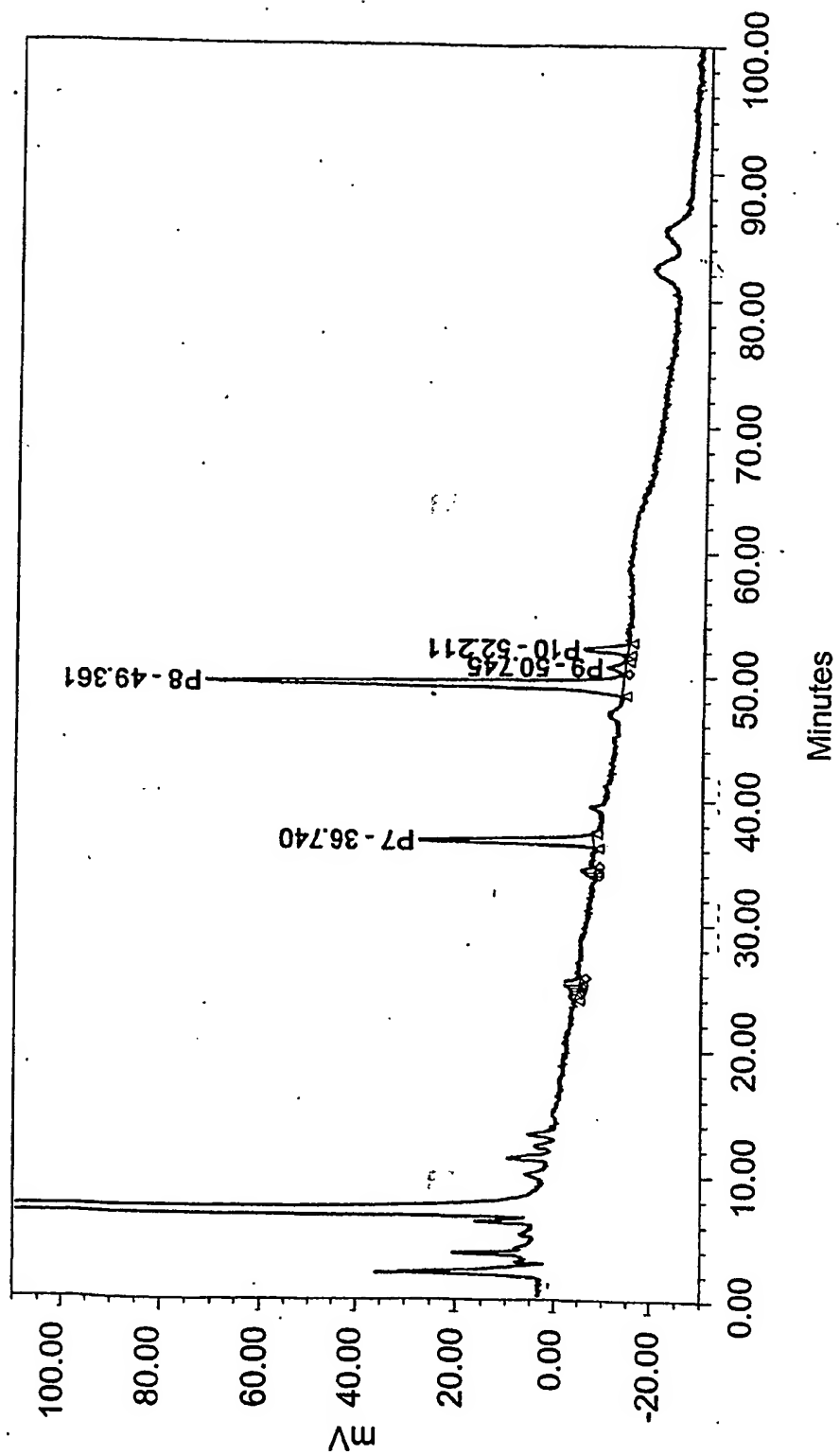


FIG. 113C

400/498

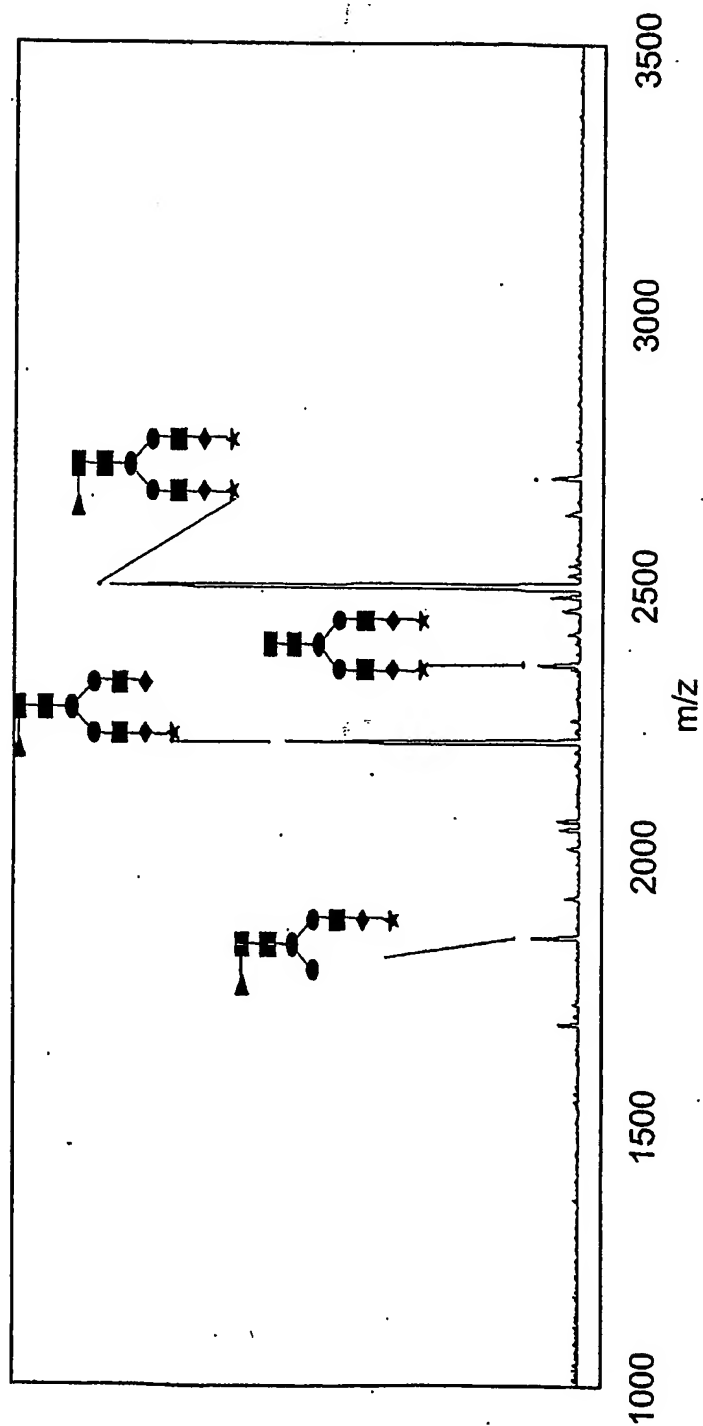


FIG. 114A

401/498

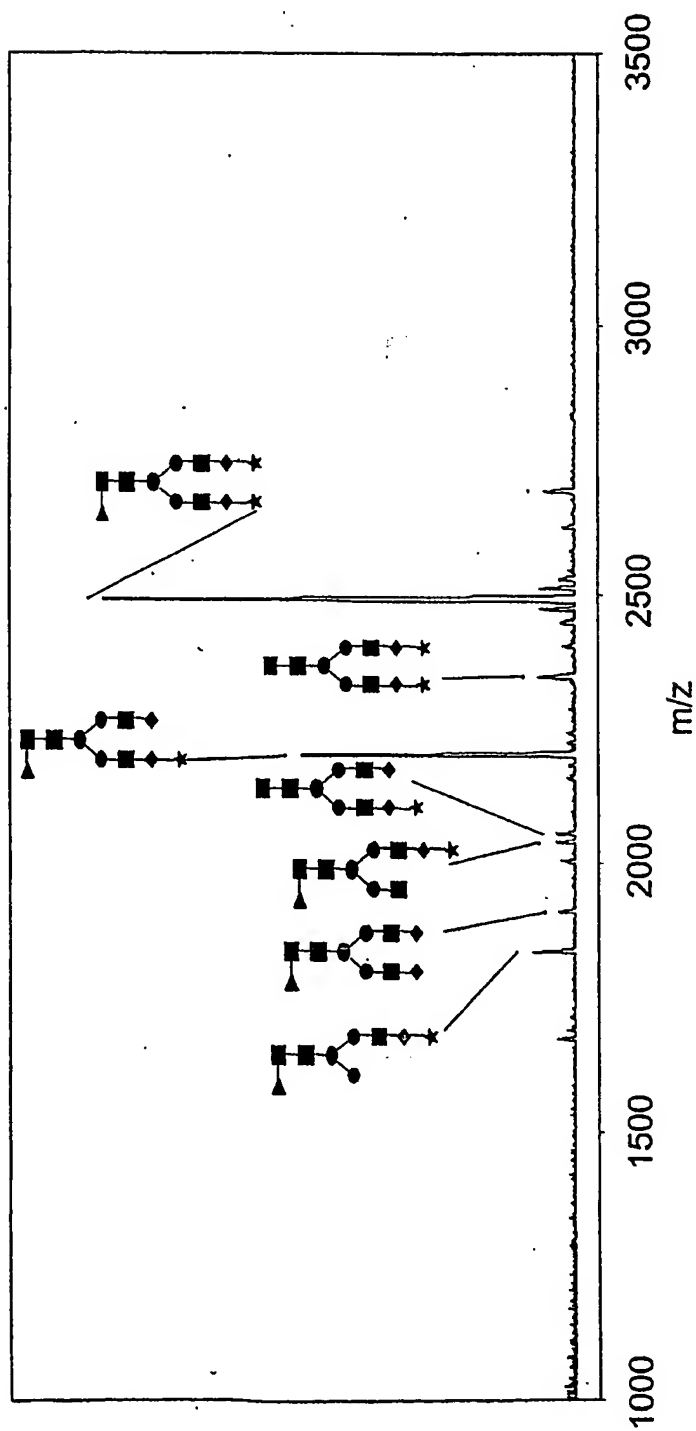


FIG. 114B



402/498

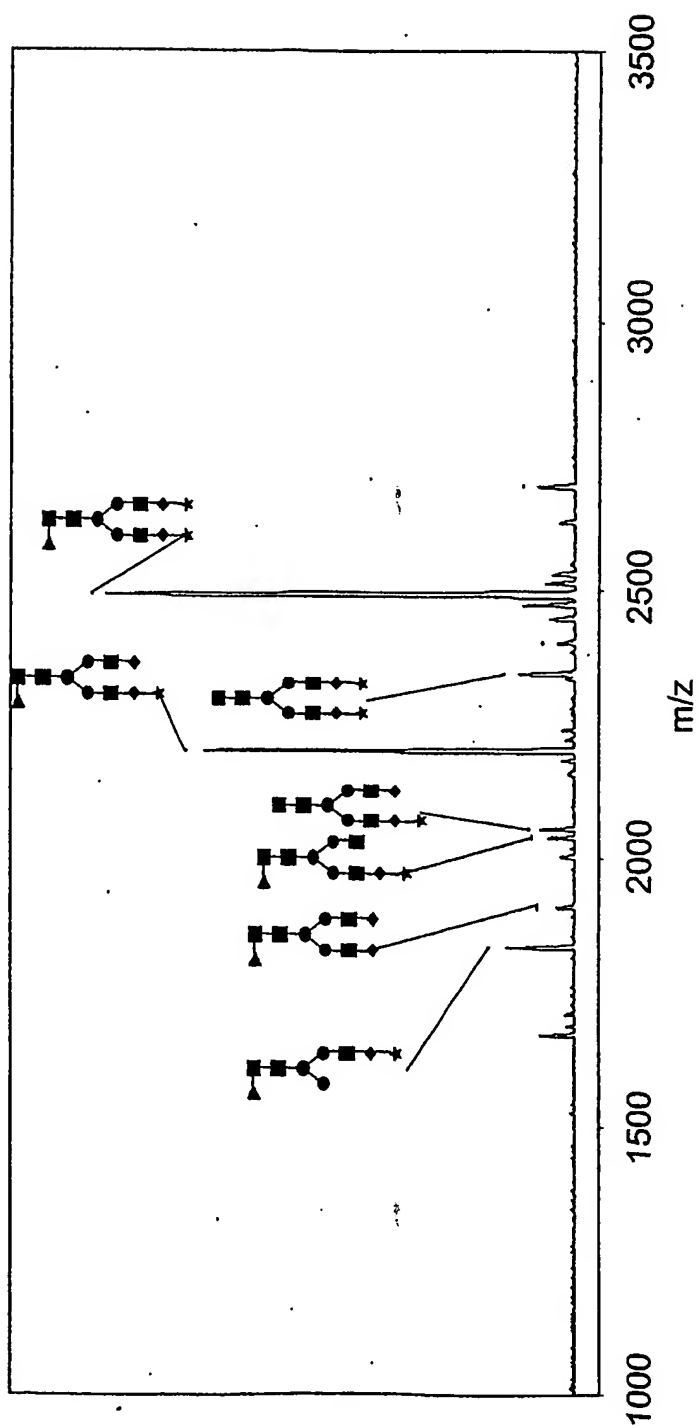


FIG. 114C

403/498

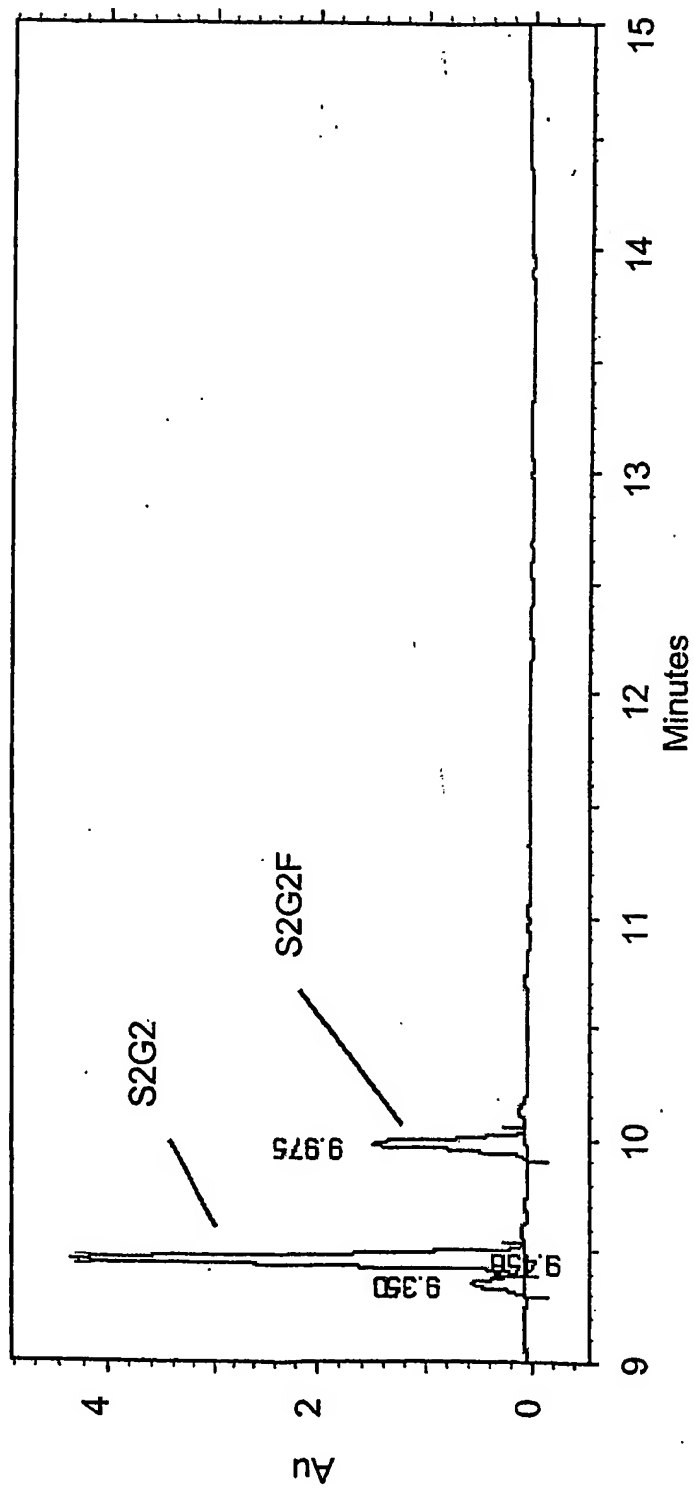


FIG. 115A

404/498

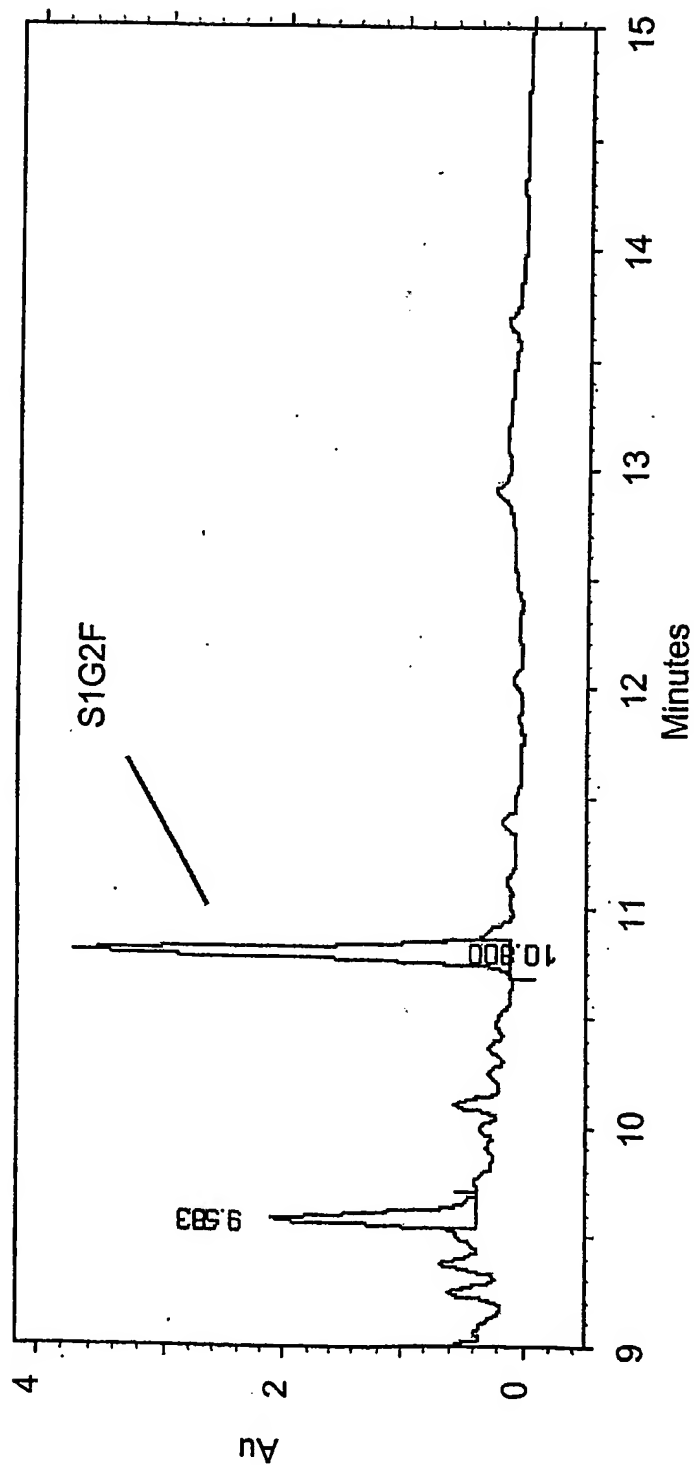


FIG. 115B

405/498

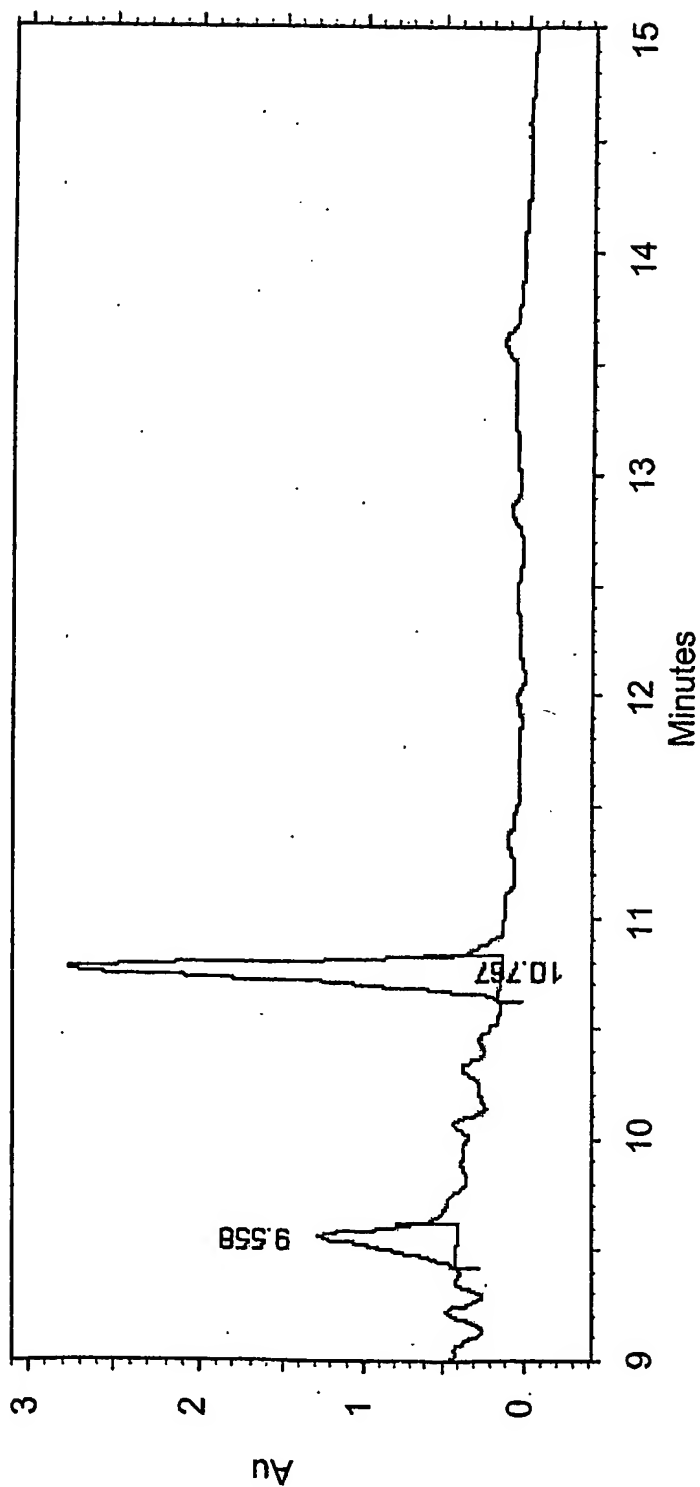


FIG. 115C

406/498

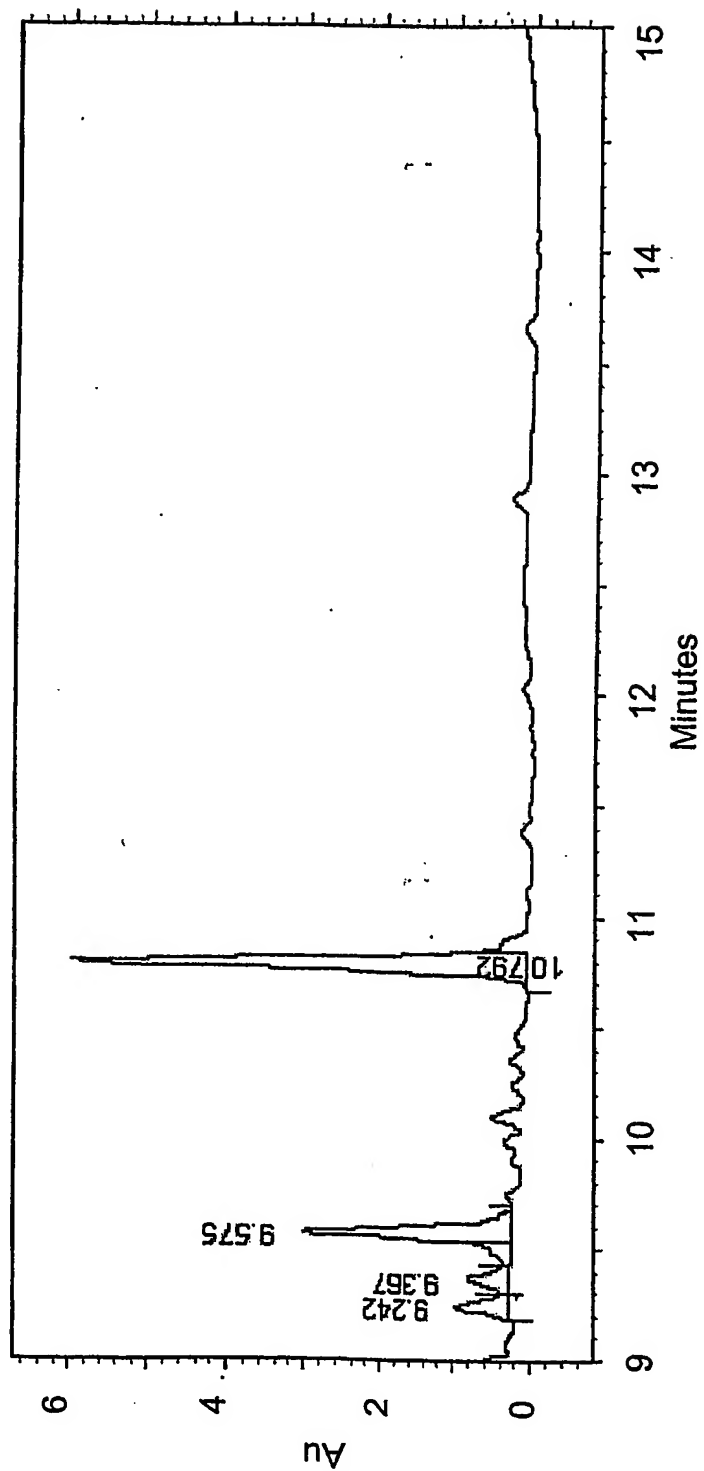


FIG. 115D

407/498

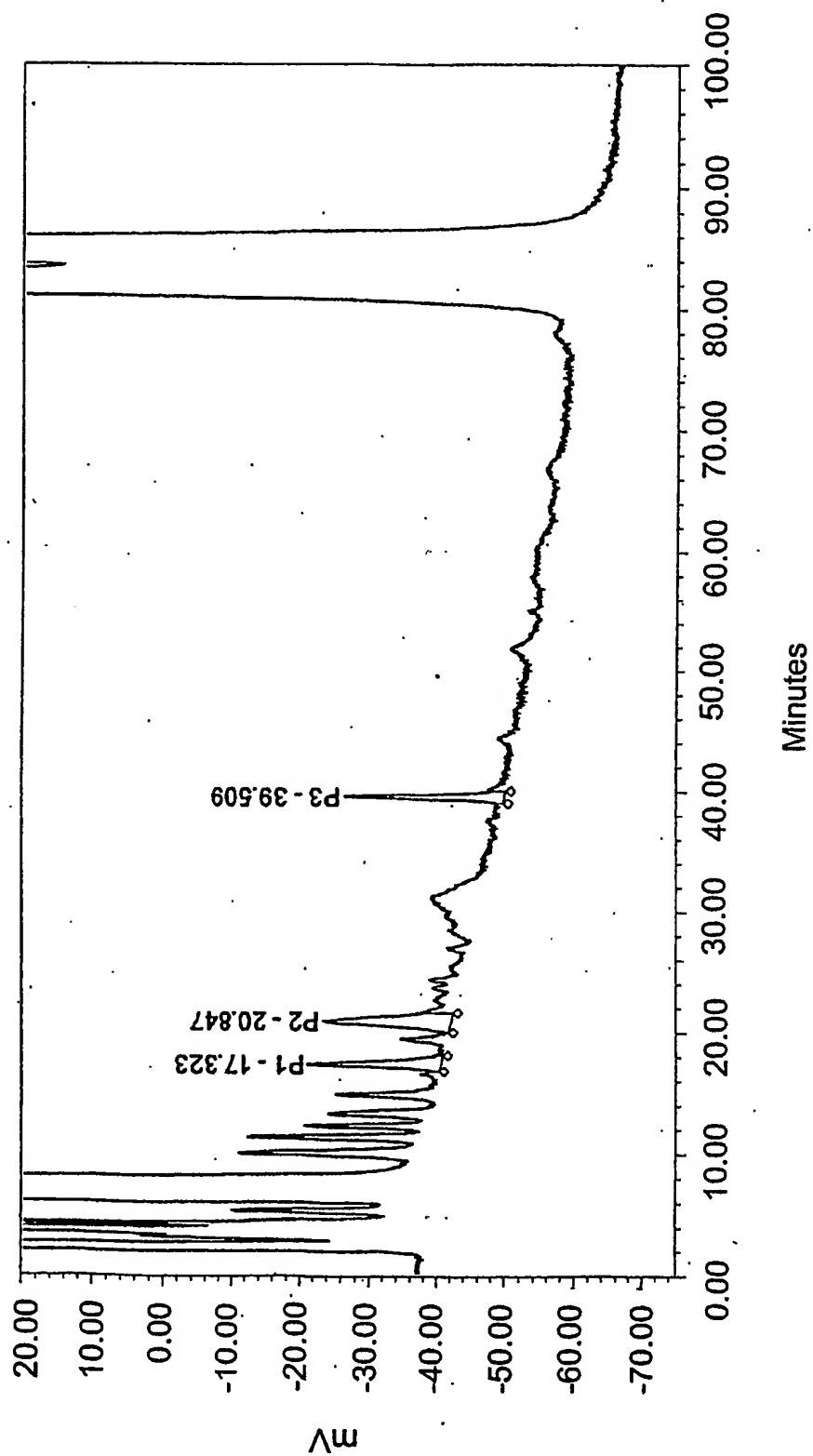


FIG. 116A

408/498

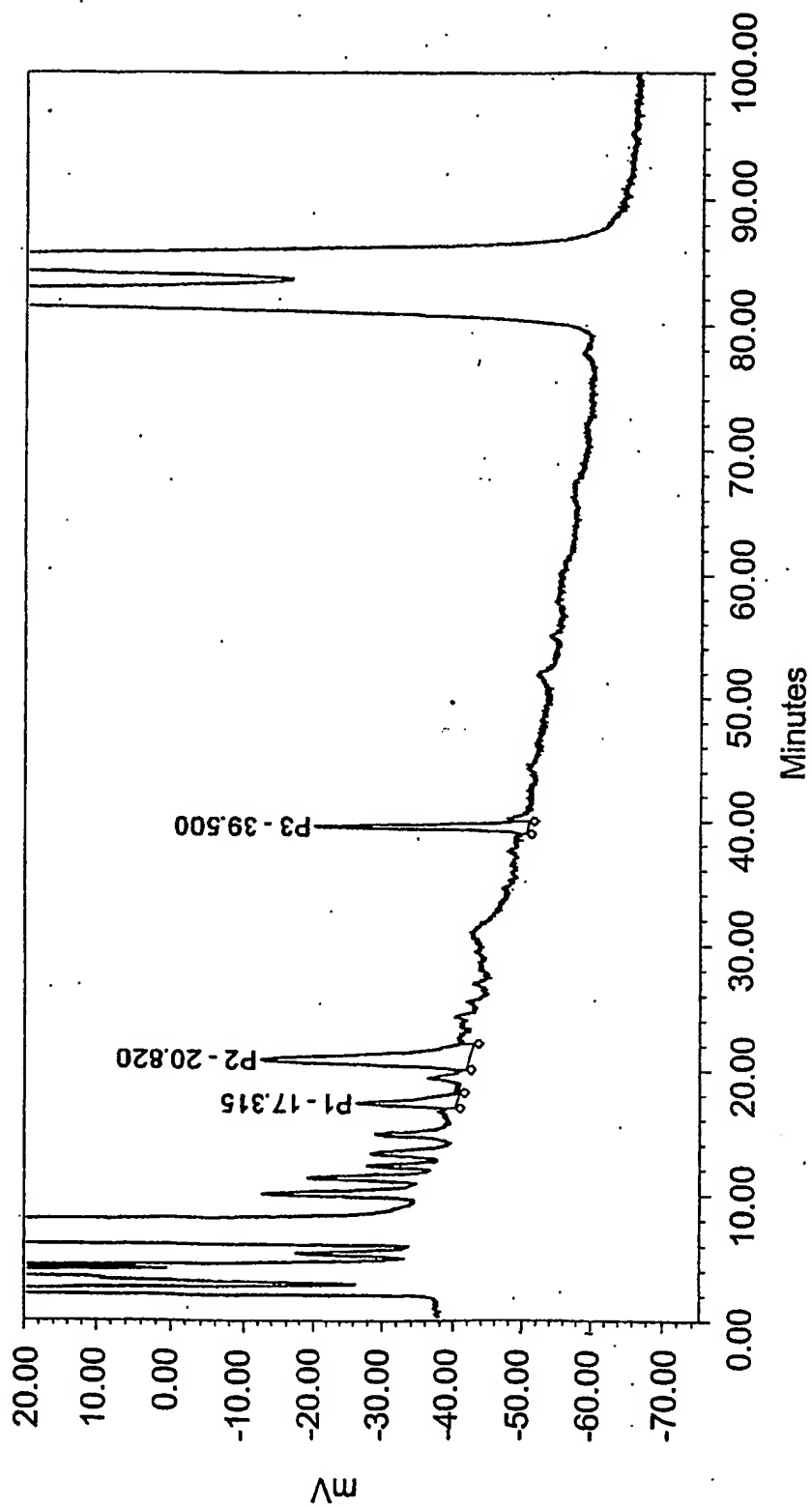


FIG. 116B

409/498

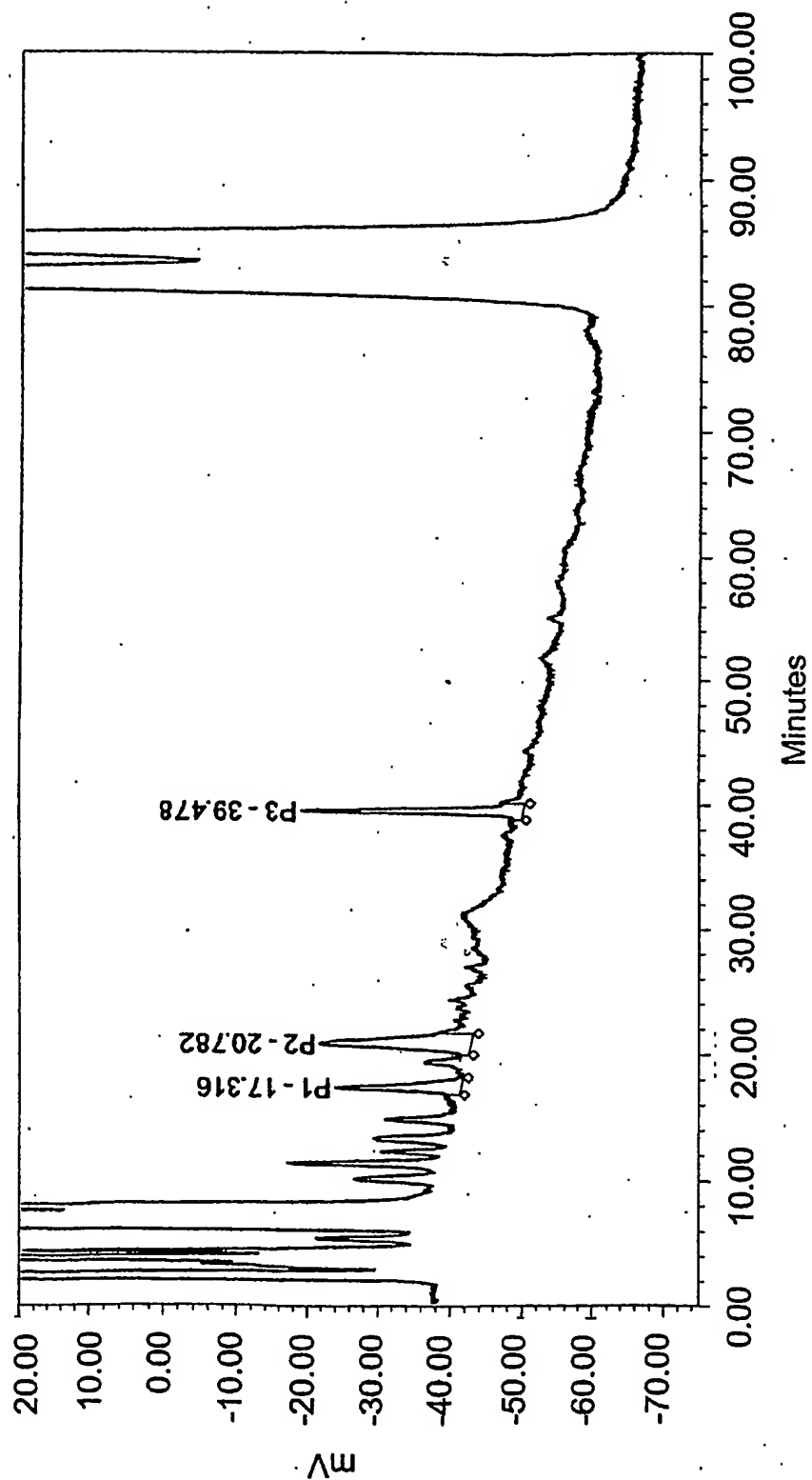


FIG. 116C



410/498

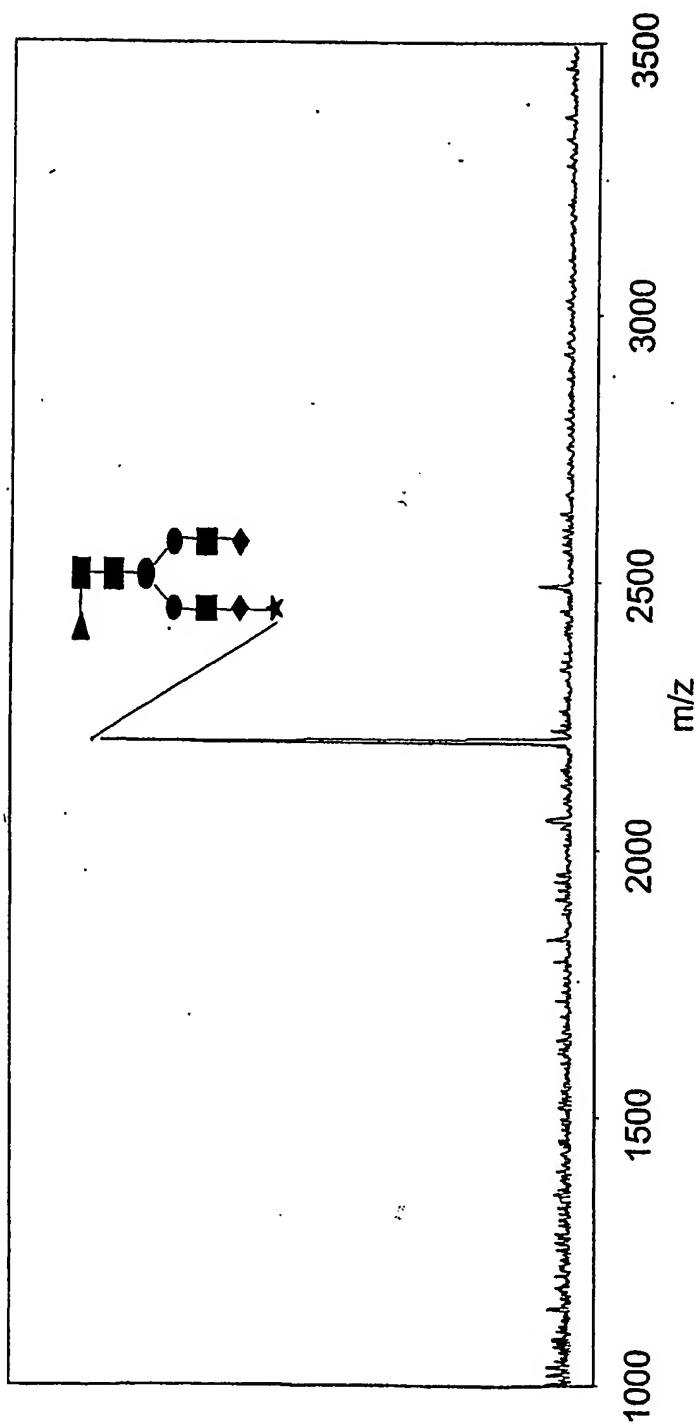


FIG. 117A

411/498

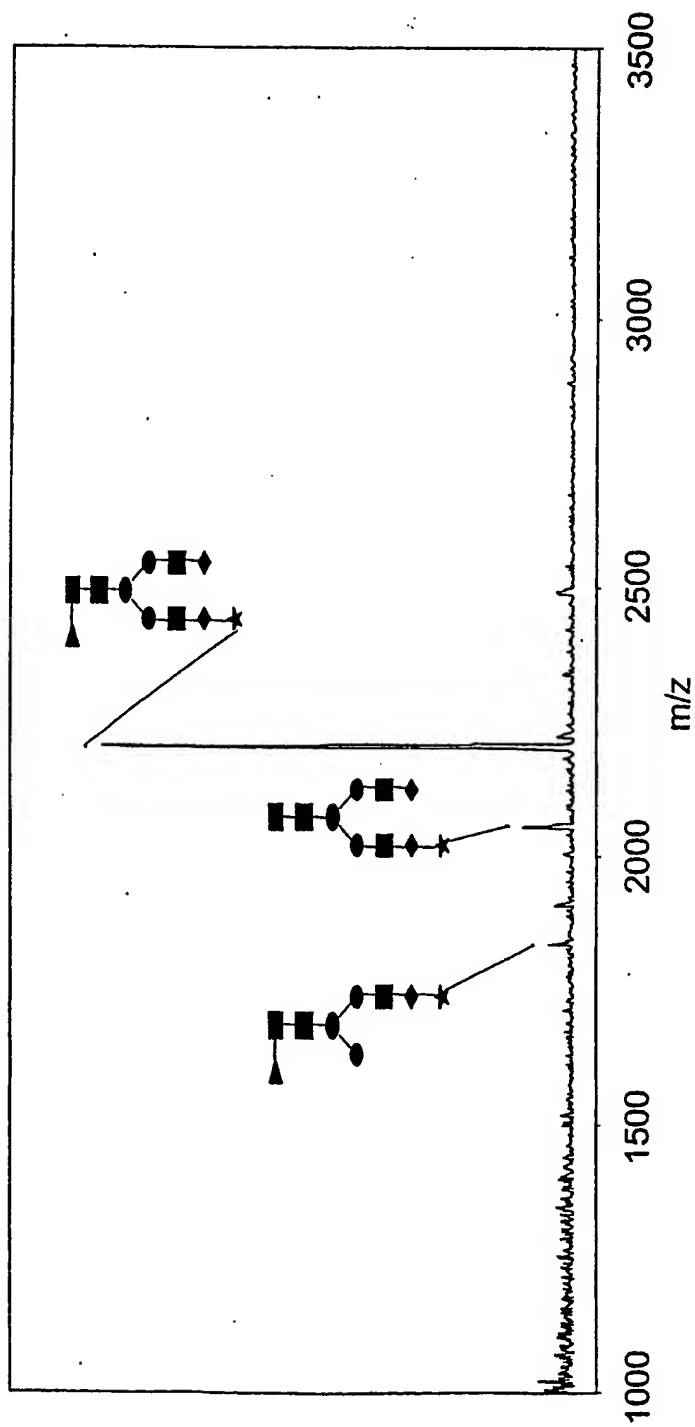


FIG. 117B

412/498

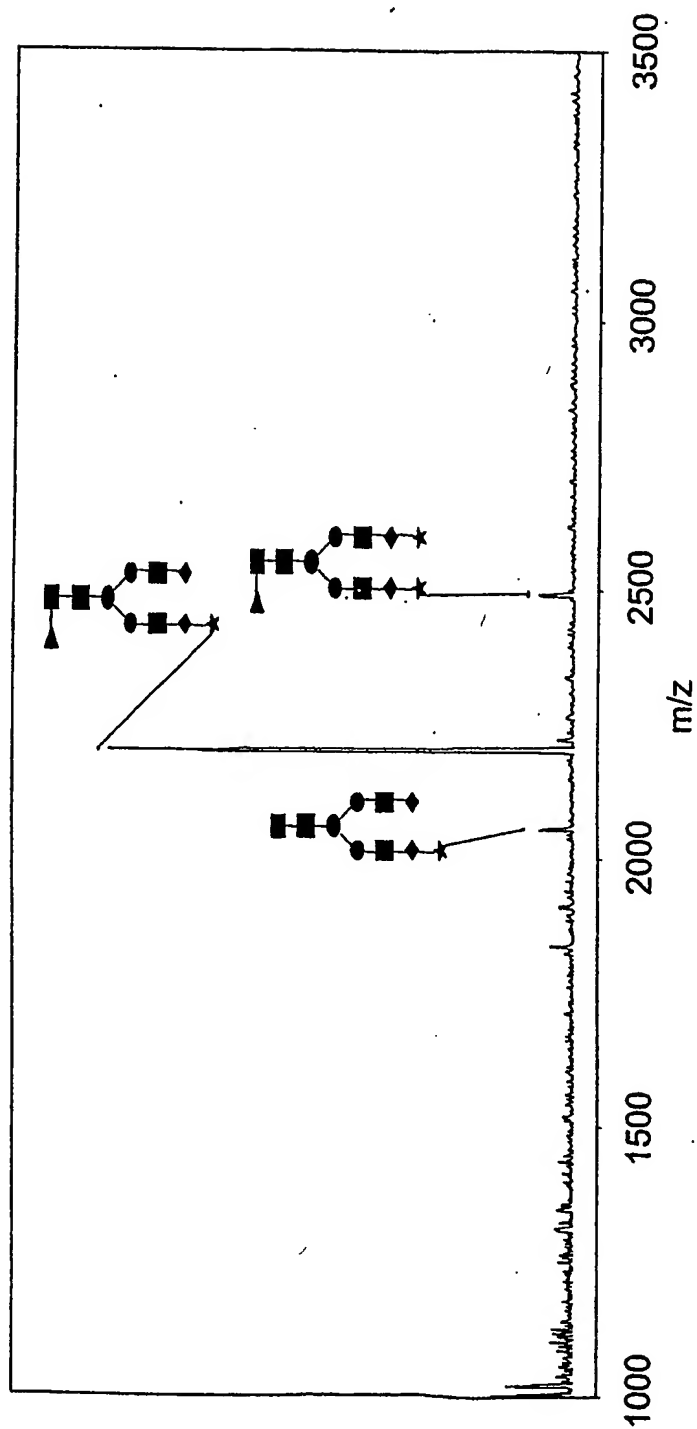


FIG. 117C



414/498

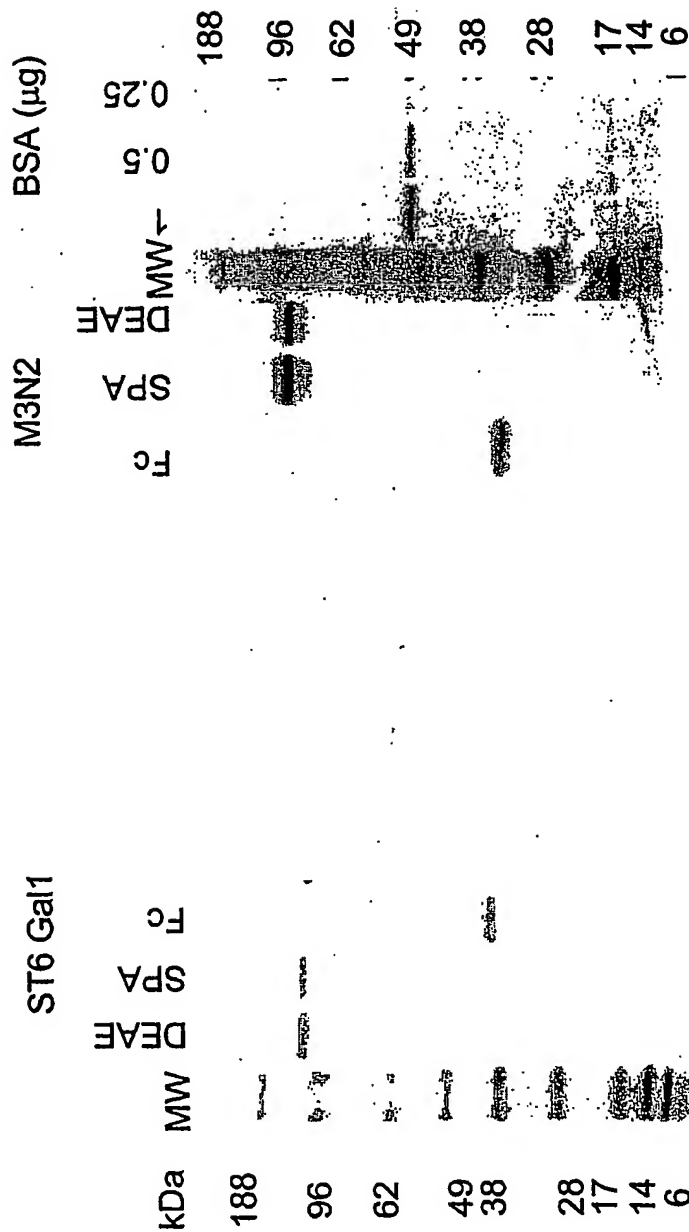


FIG. 118D

FIG. 118C

415/498

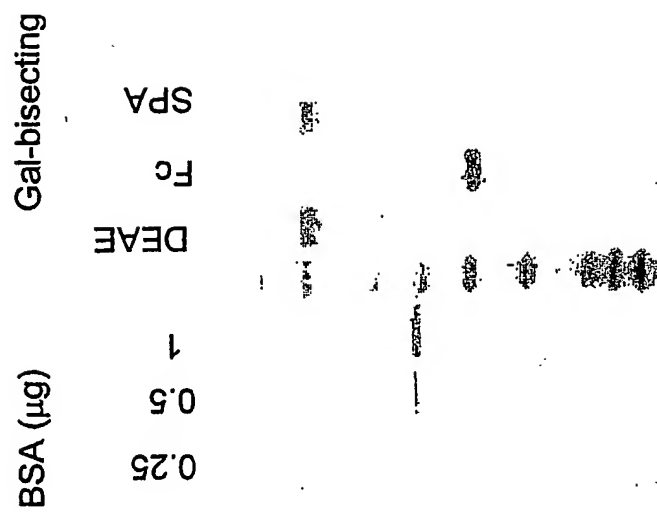


FIG. 118E

416/498

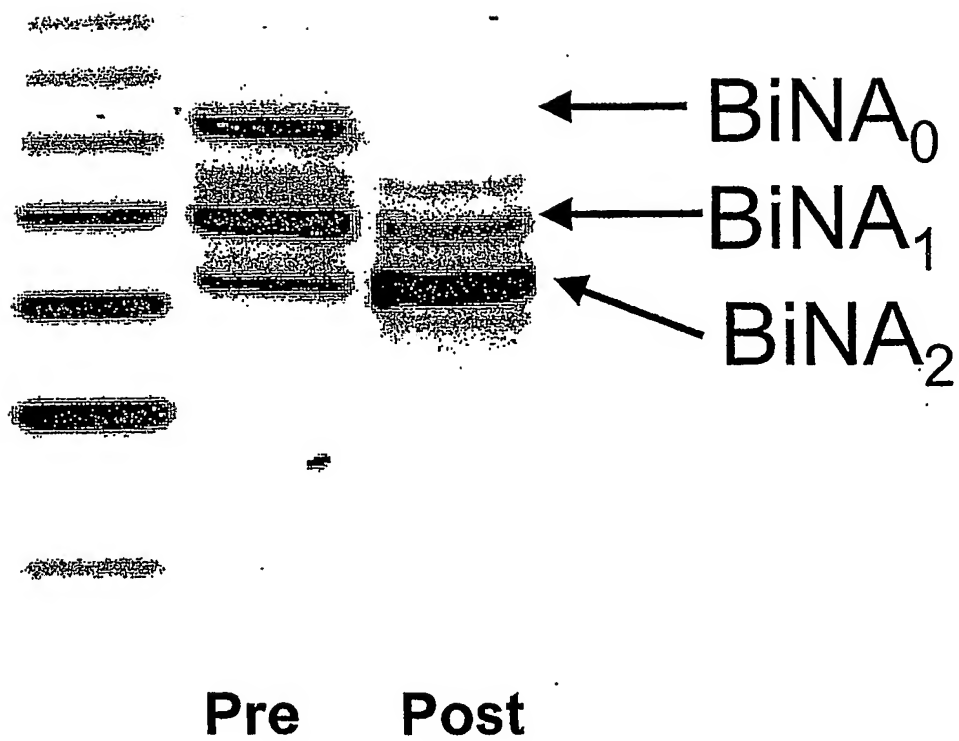


FIG. 119

417/498

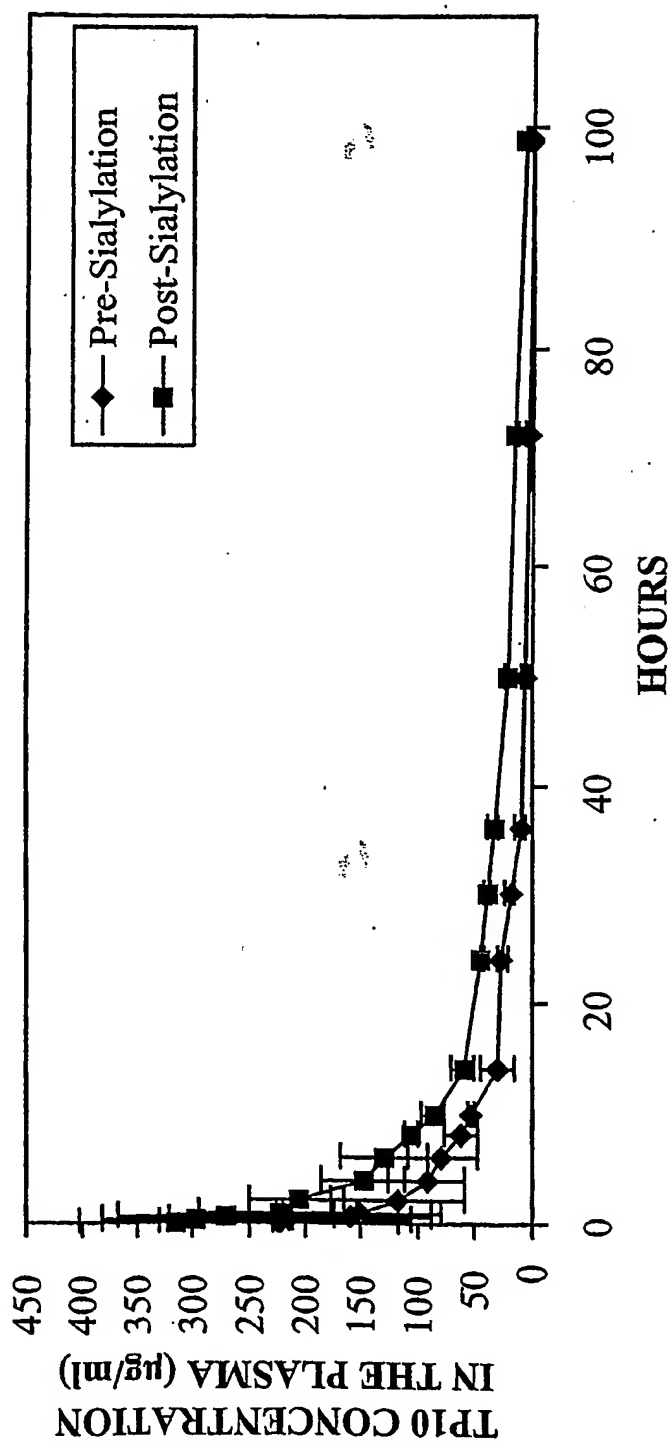


FIG. 120



418/498

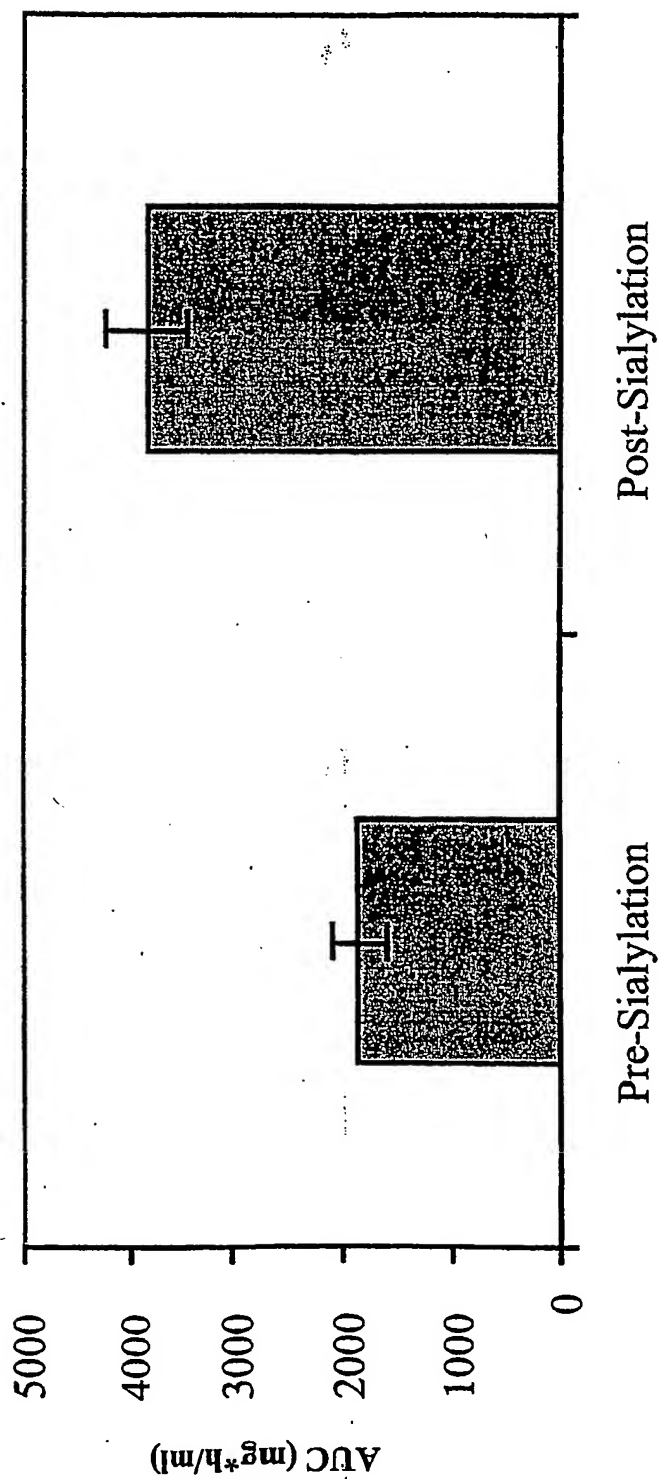
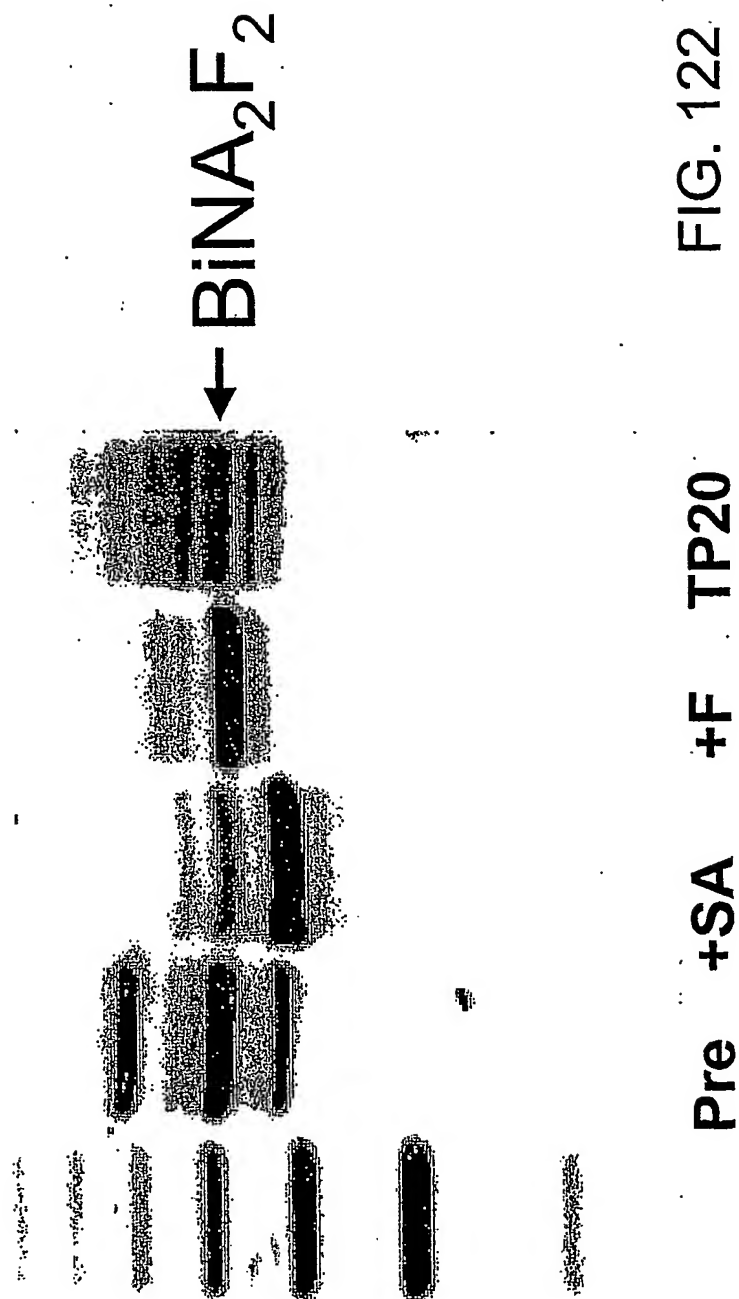


FIG. 121

419/498



420/498

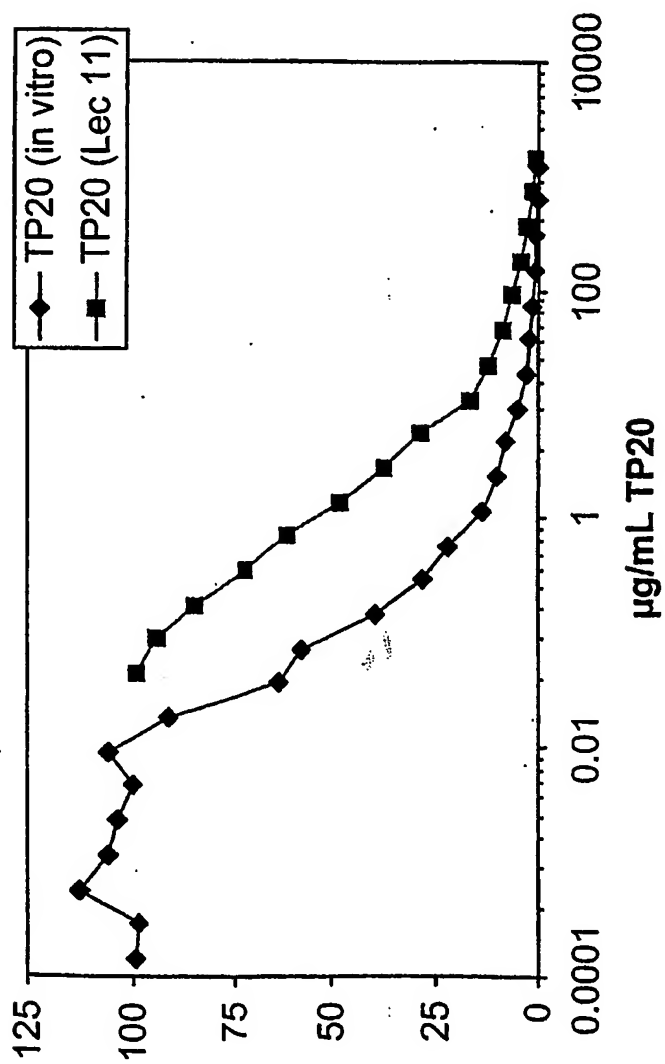


FIG. 123

421/498

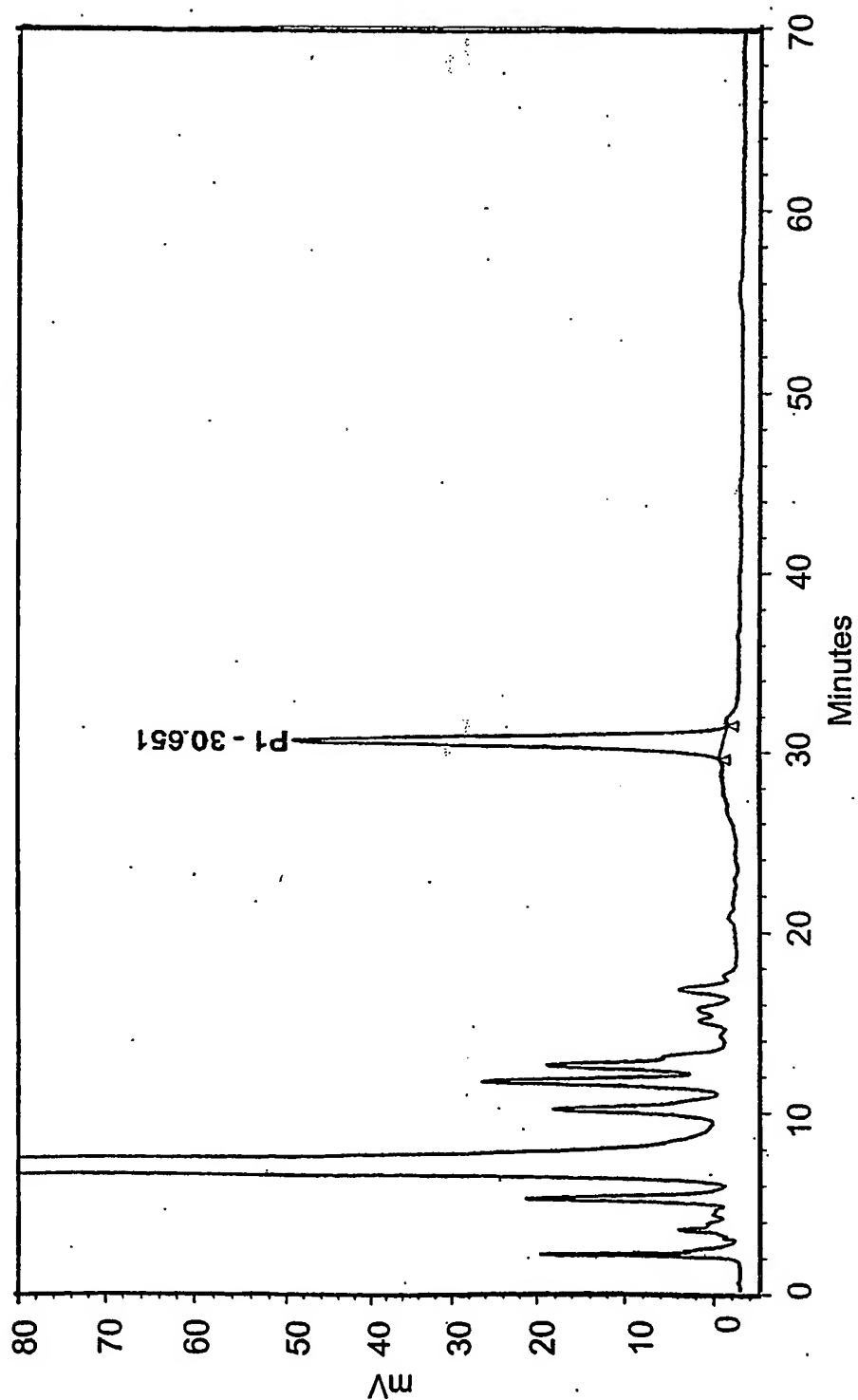


FIG. 124

422/498

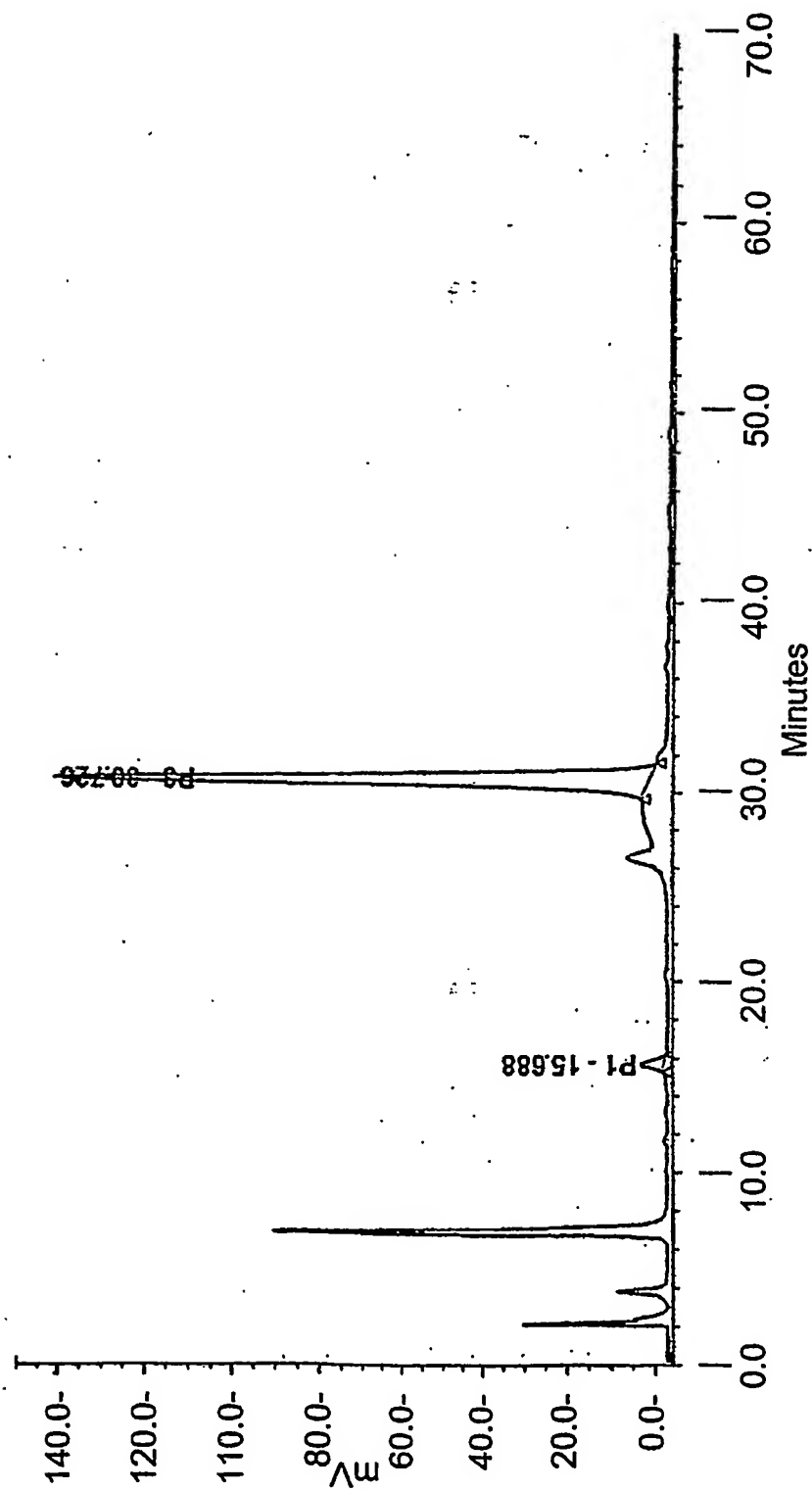


FIG. 125A

423/498

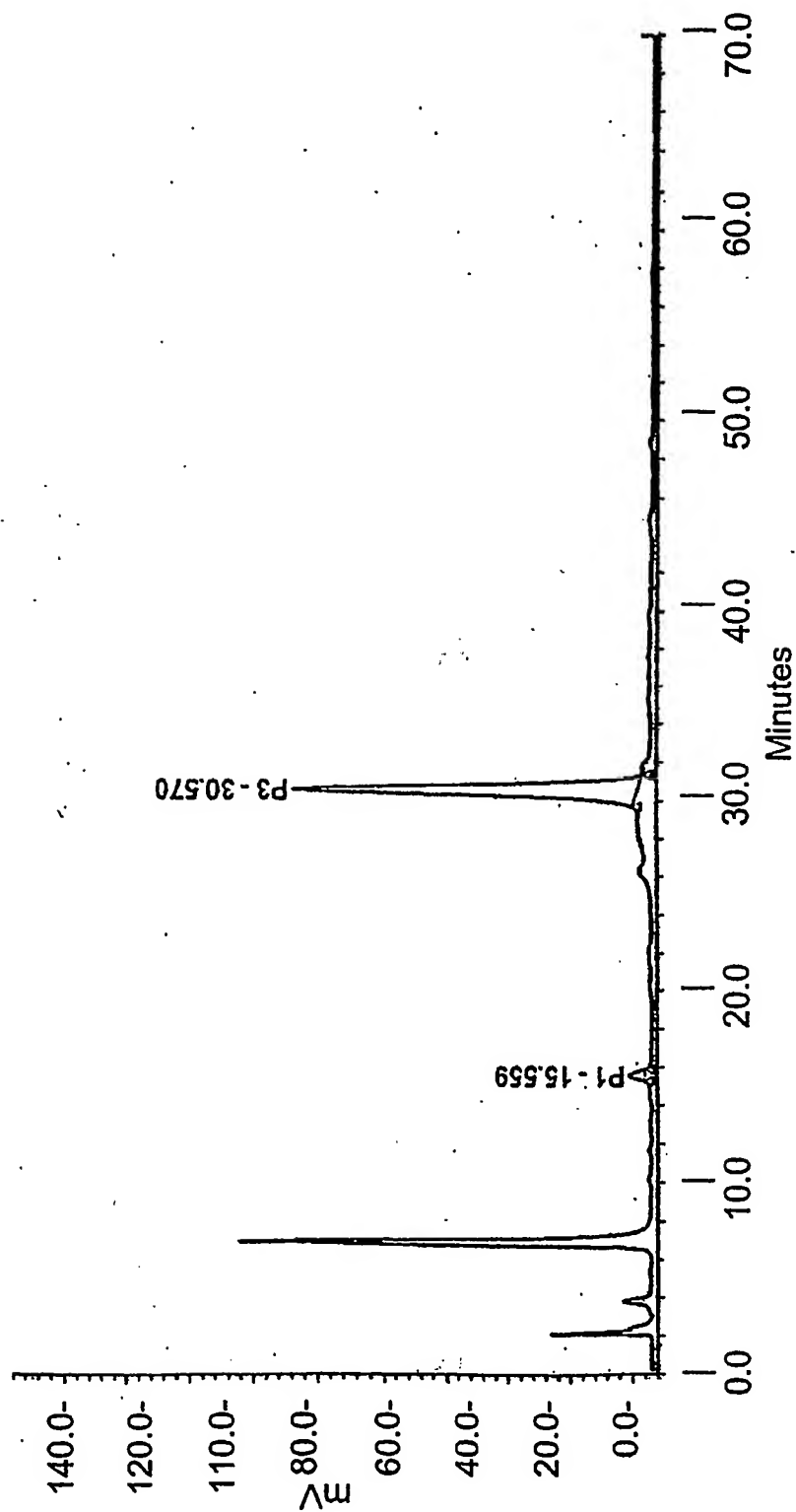


FIG. 125B

424/498

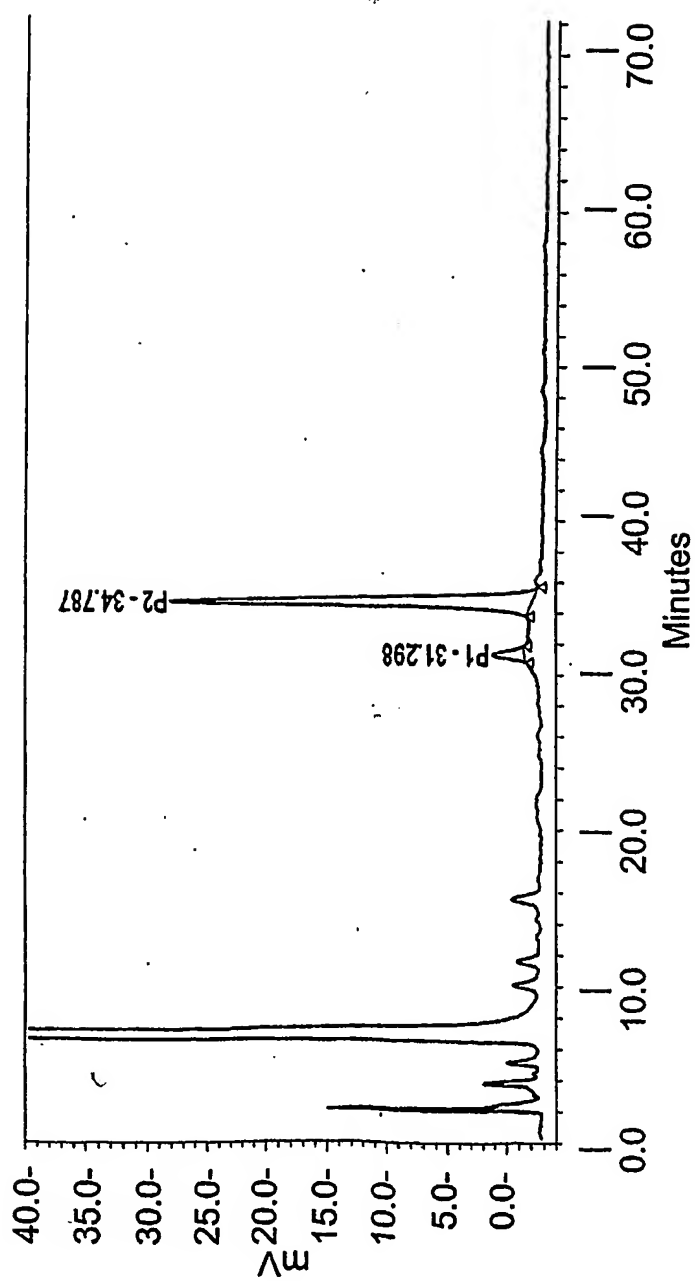


FIG. 126

425/498

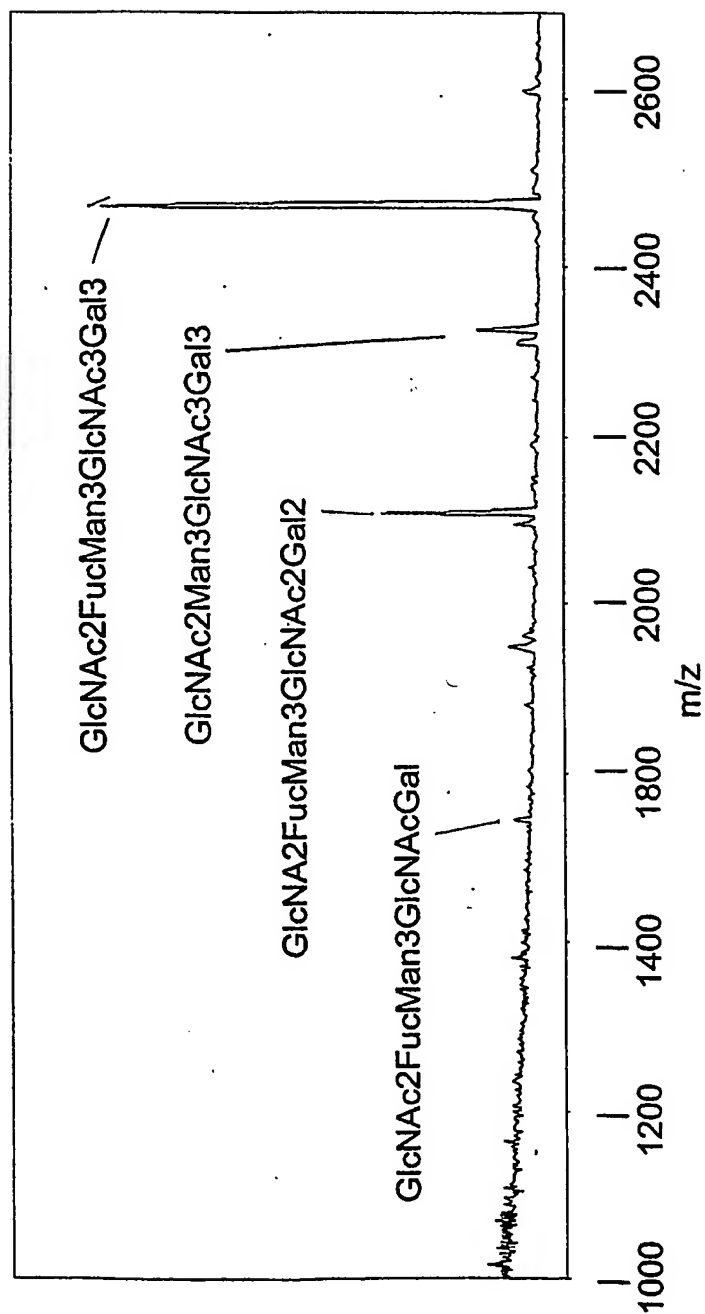


FIG. 127



426/498

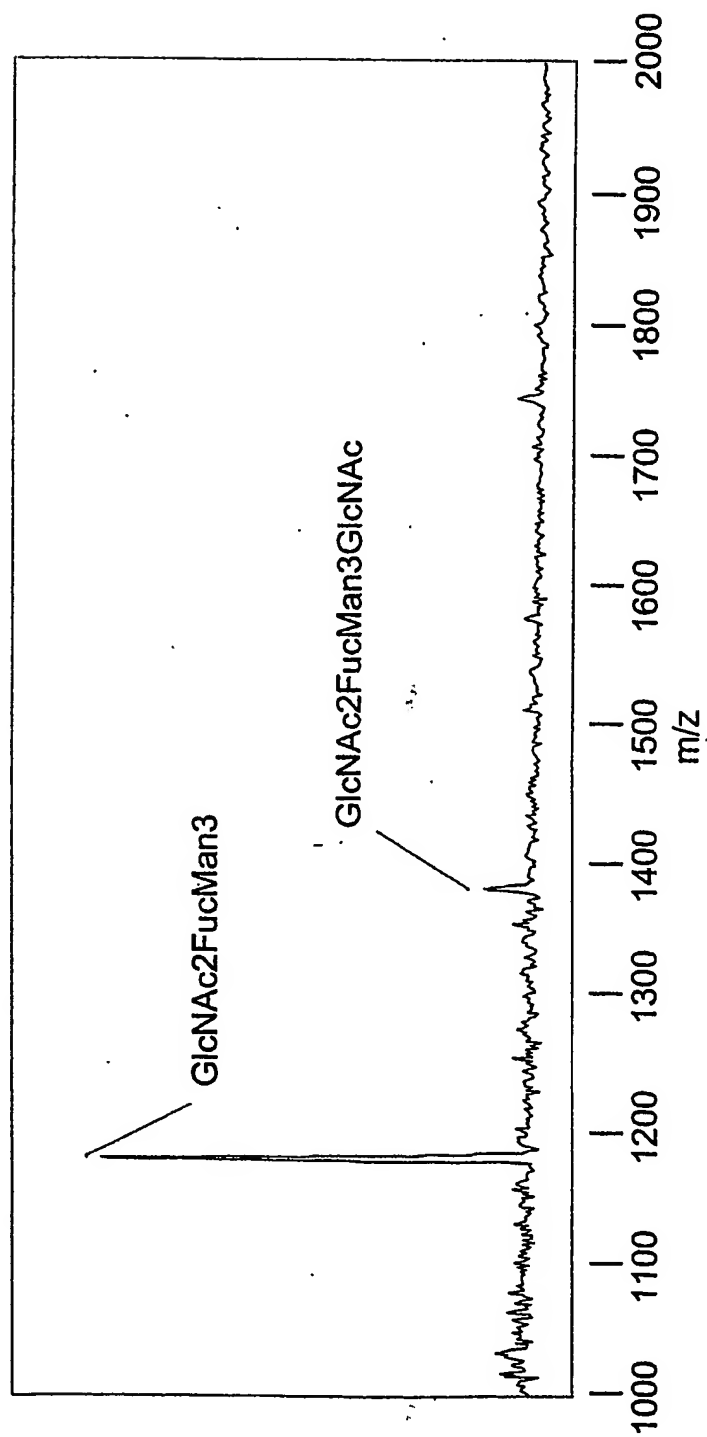


FIG. 128

427/498

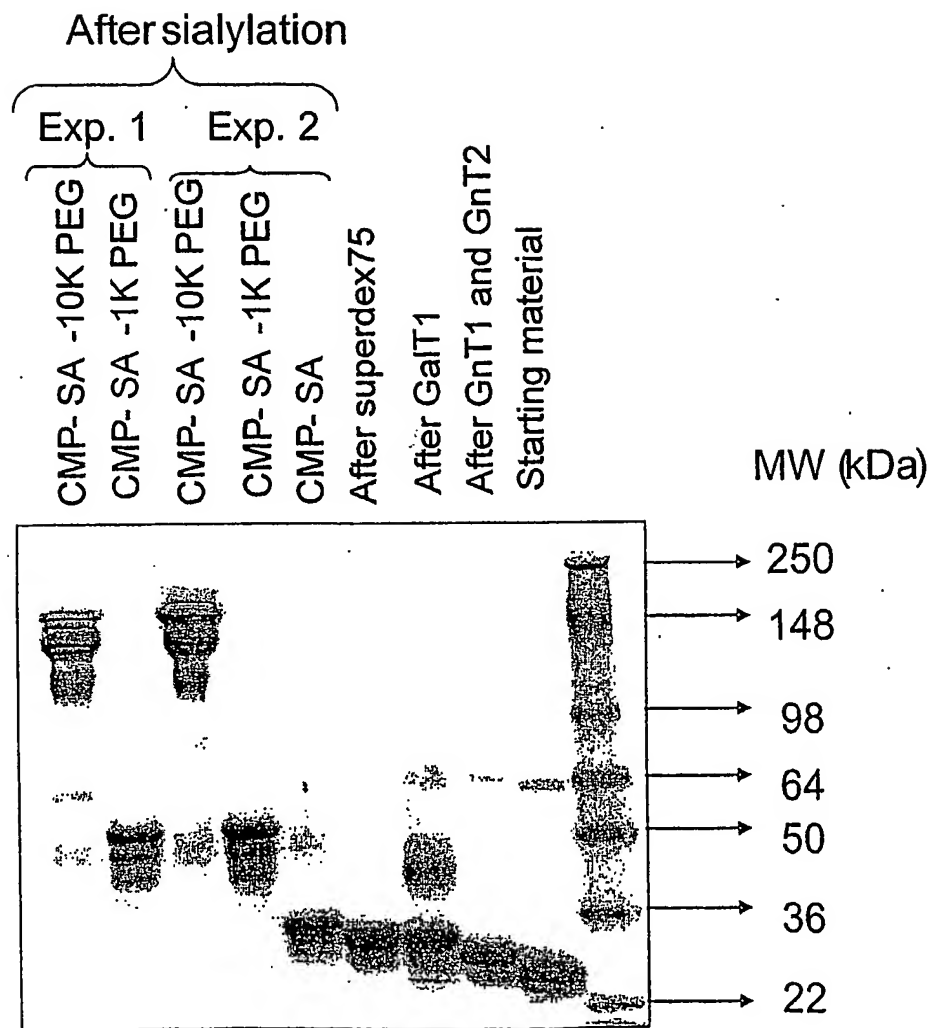


FIG. 129

428/498

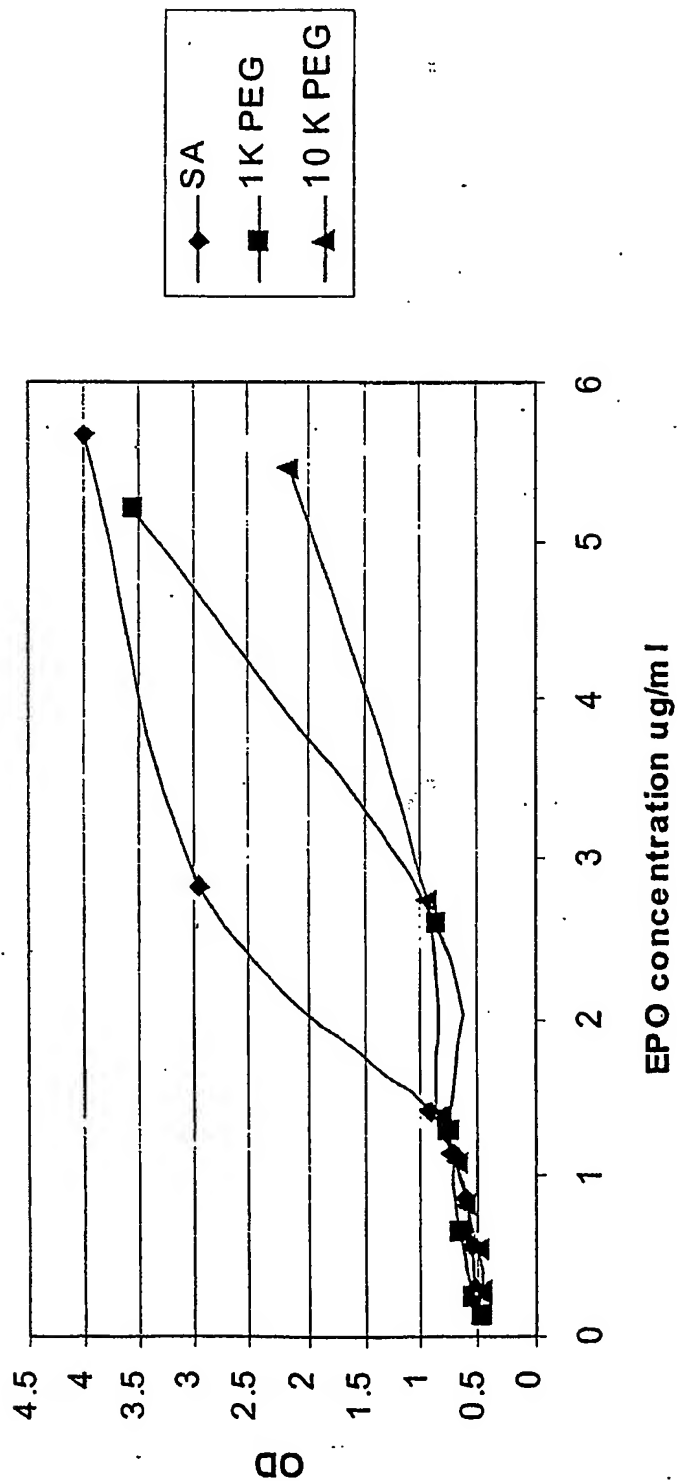
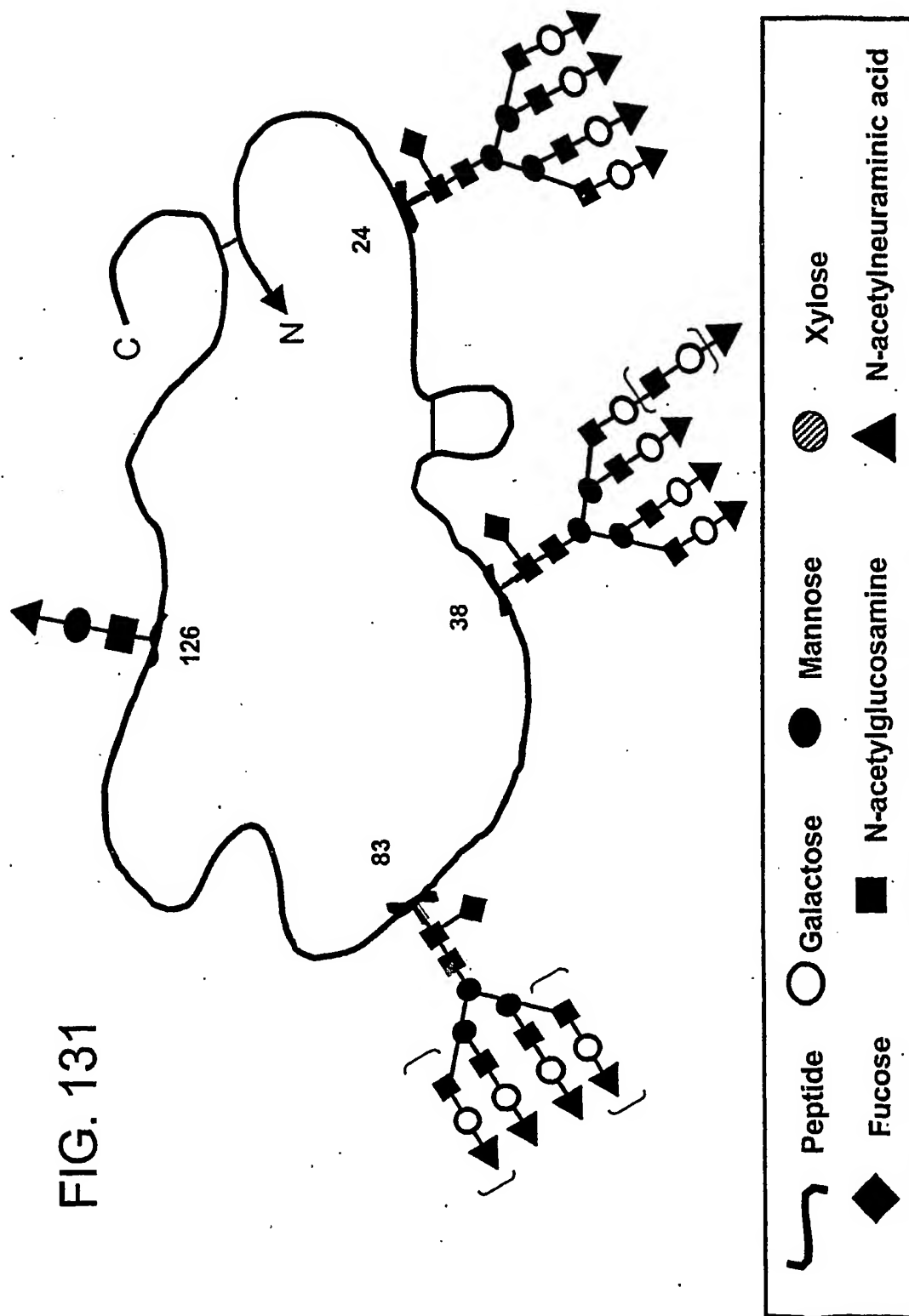


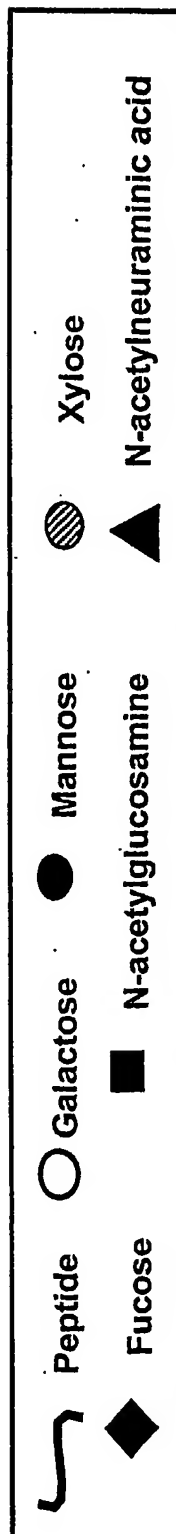
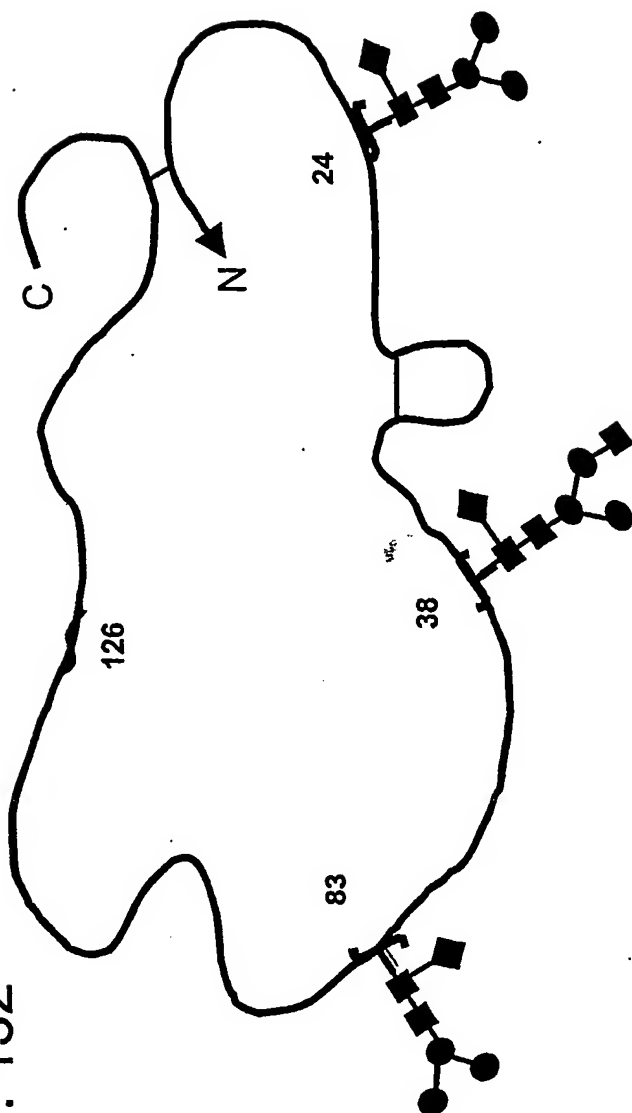
FIG. 130

429/498



430/498

FIG. 132



431/498

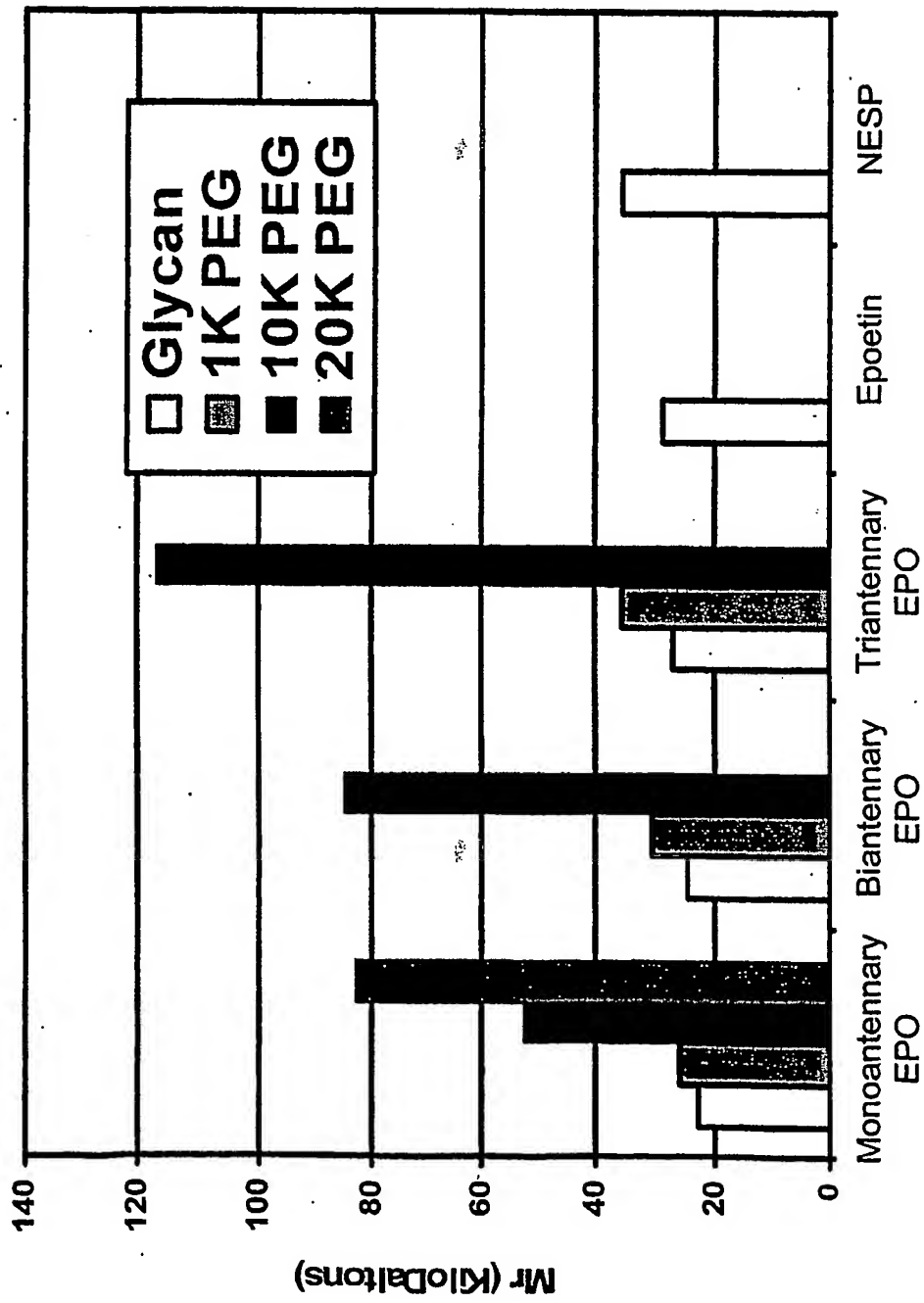
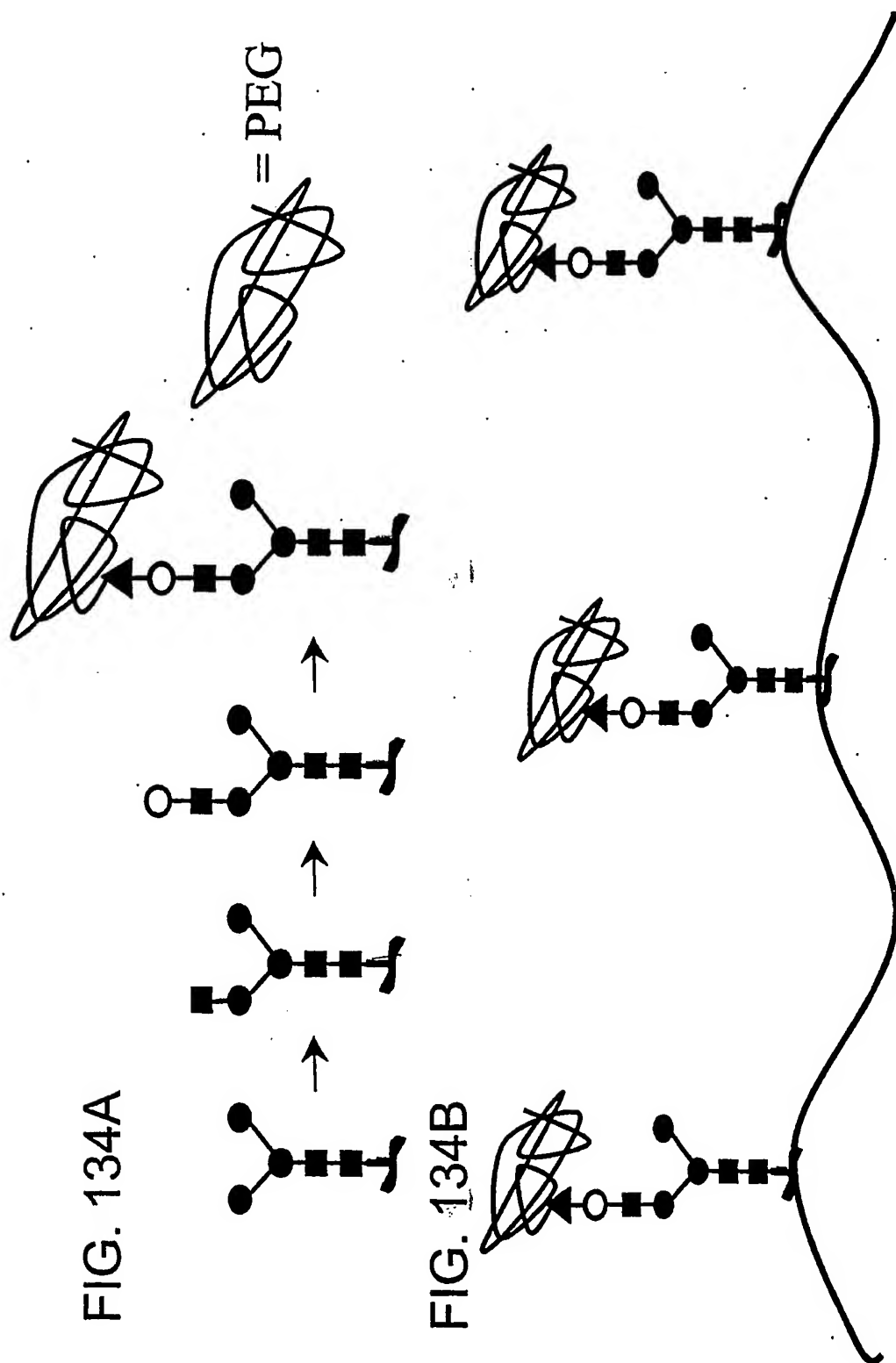


FIG. 133

432/498



433/498

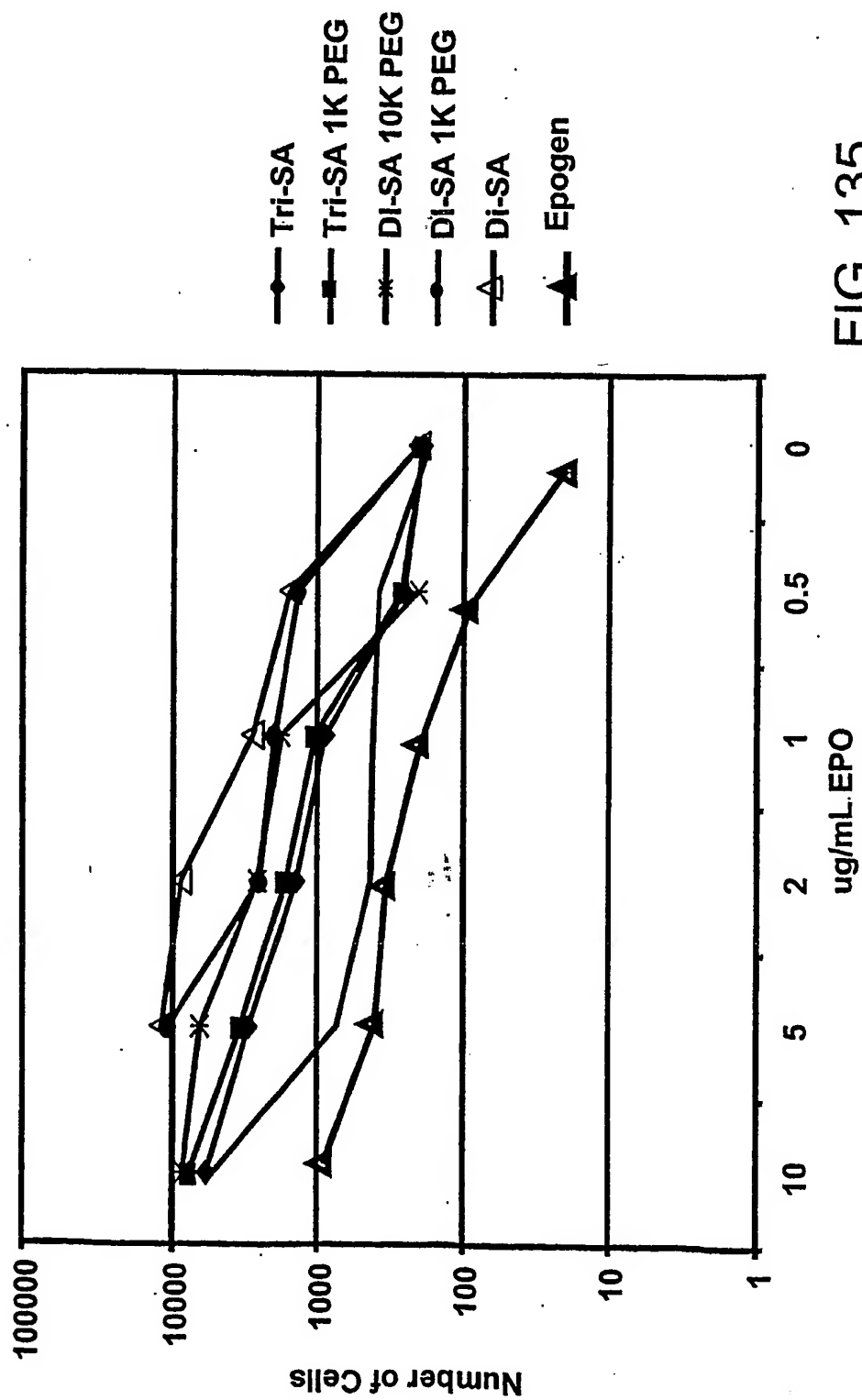


FIG. 135



434/498

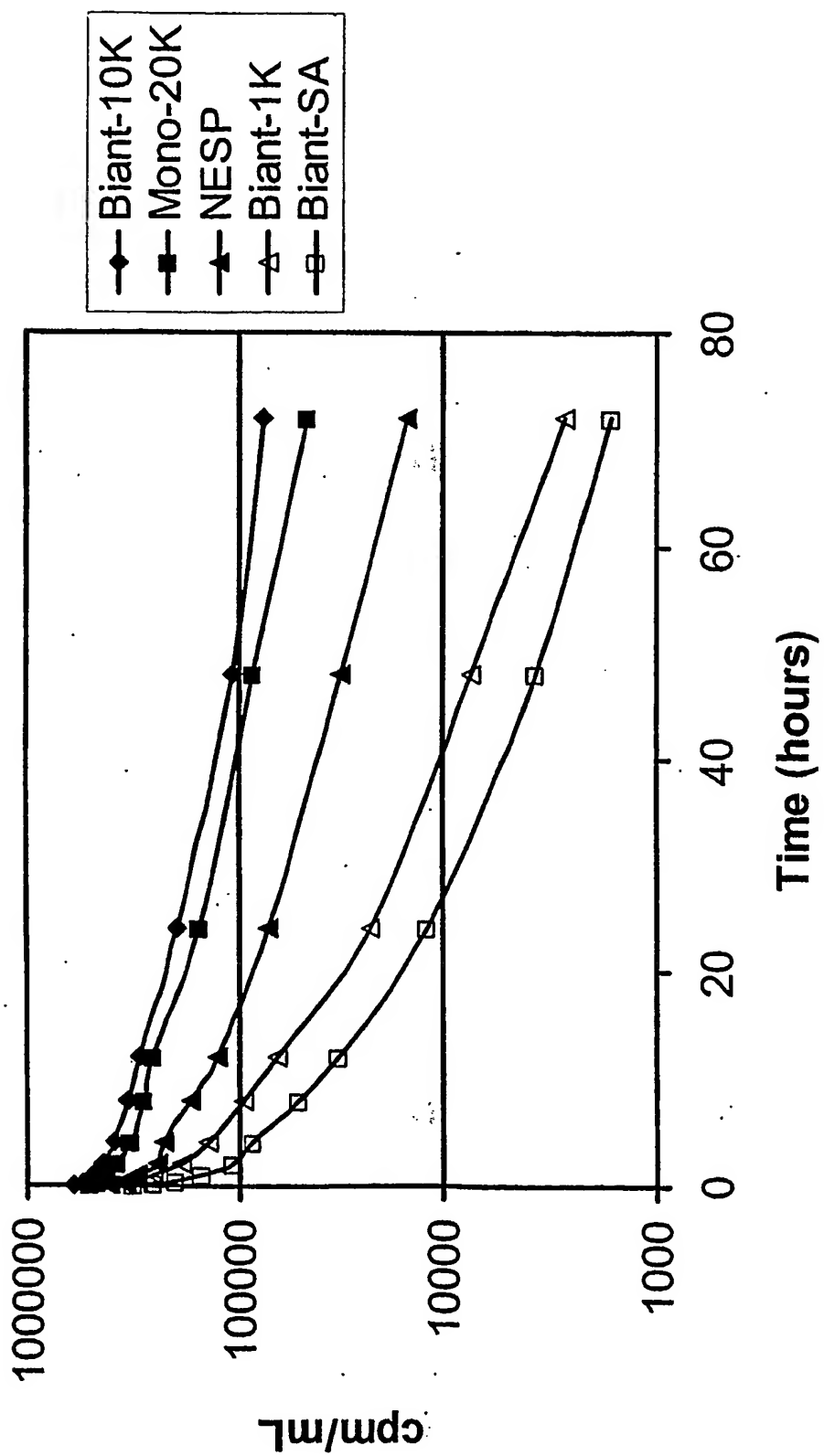


FIG. 136

435/498

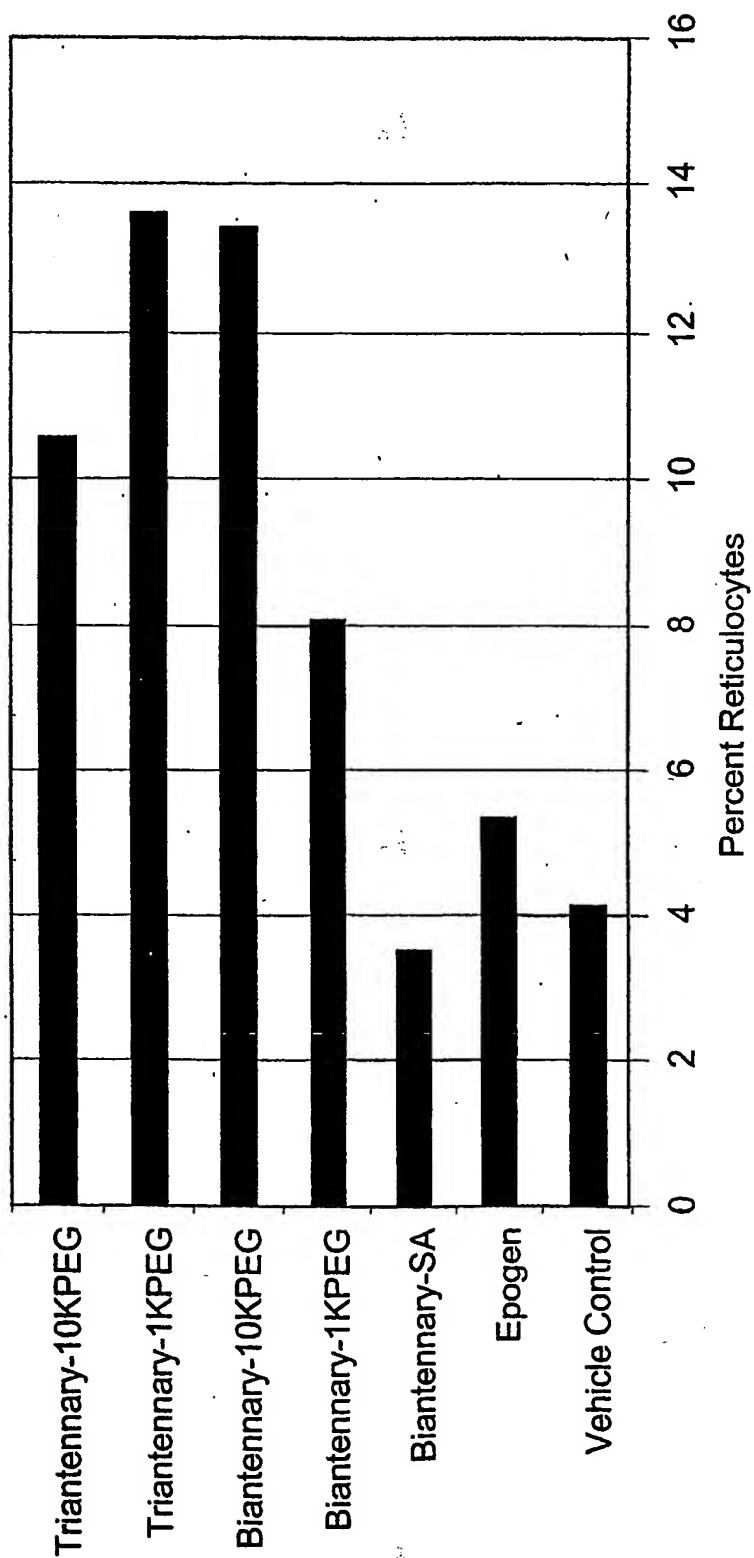


FIG. 137

436/498

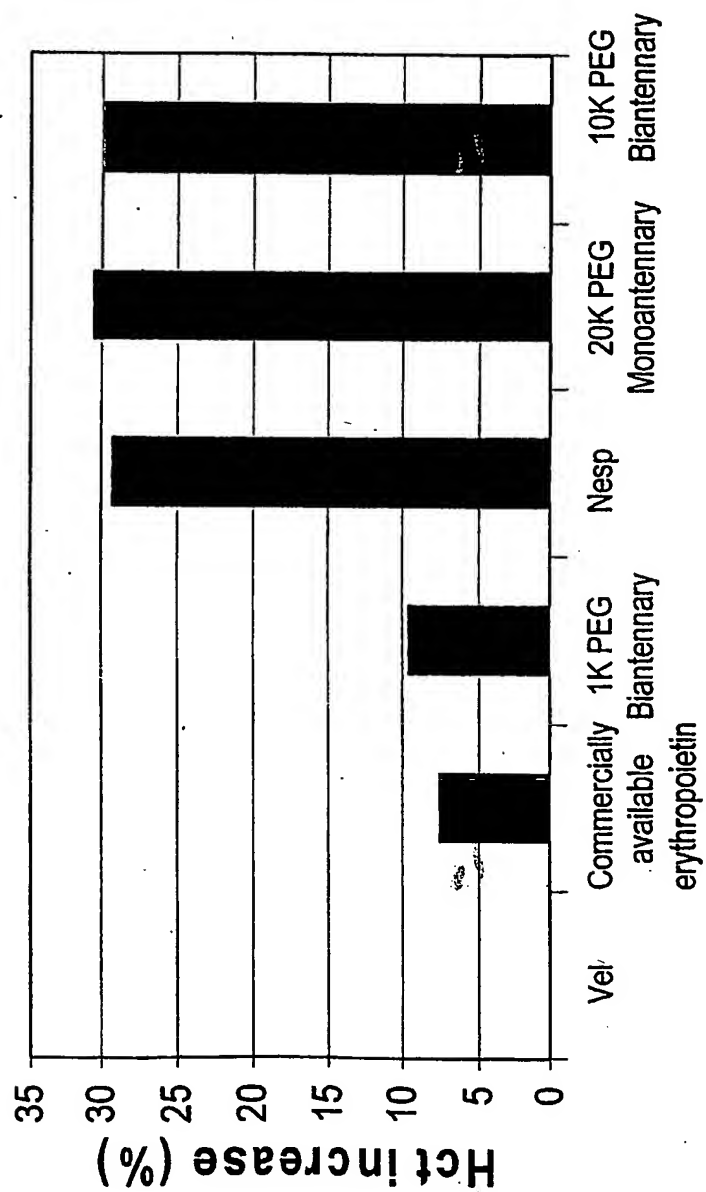


FIG. 138

437/498

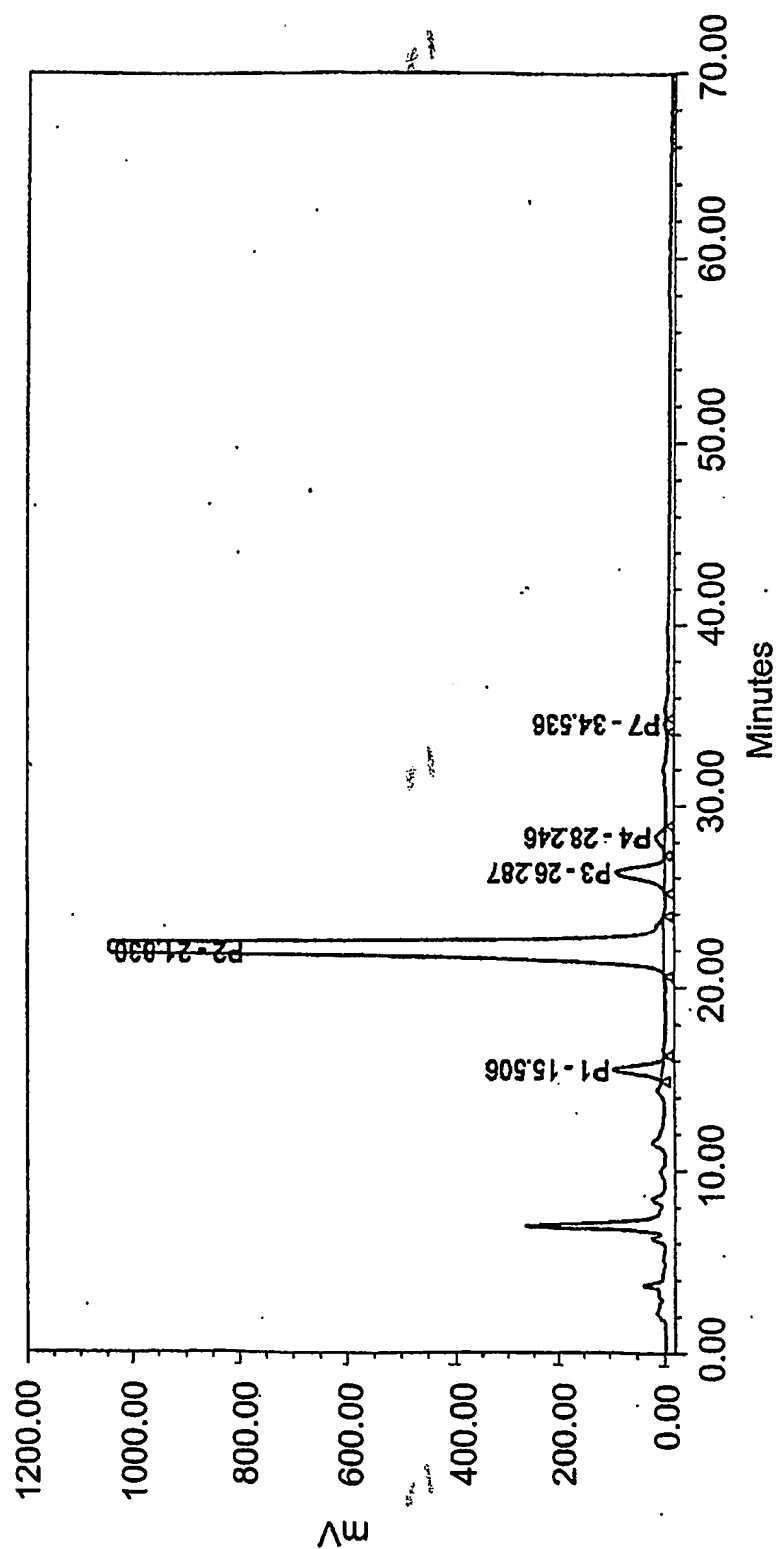


FIG. 139A

438/498

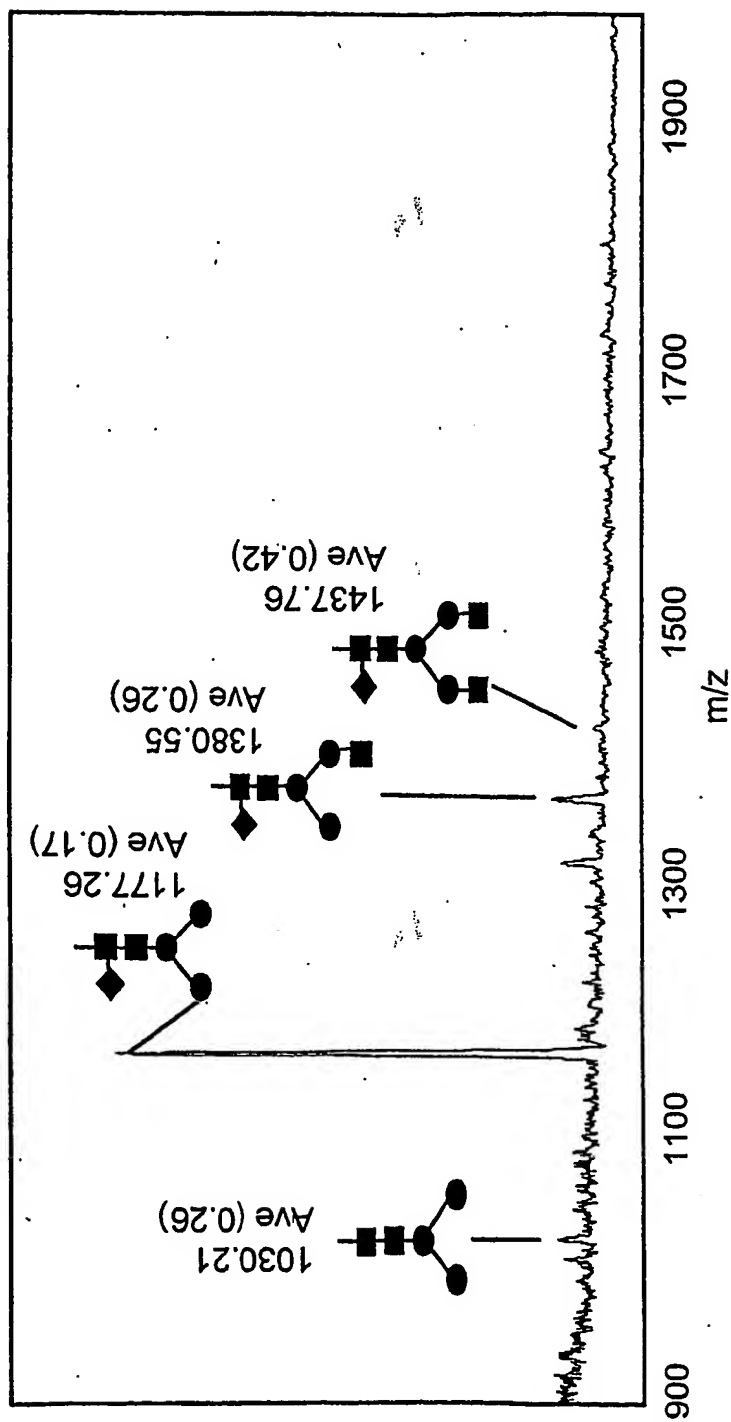


FIG. 139B

439/498

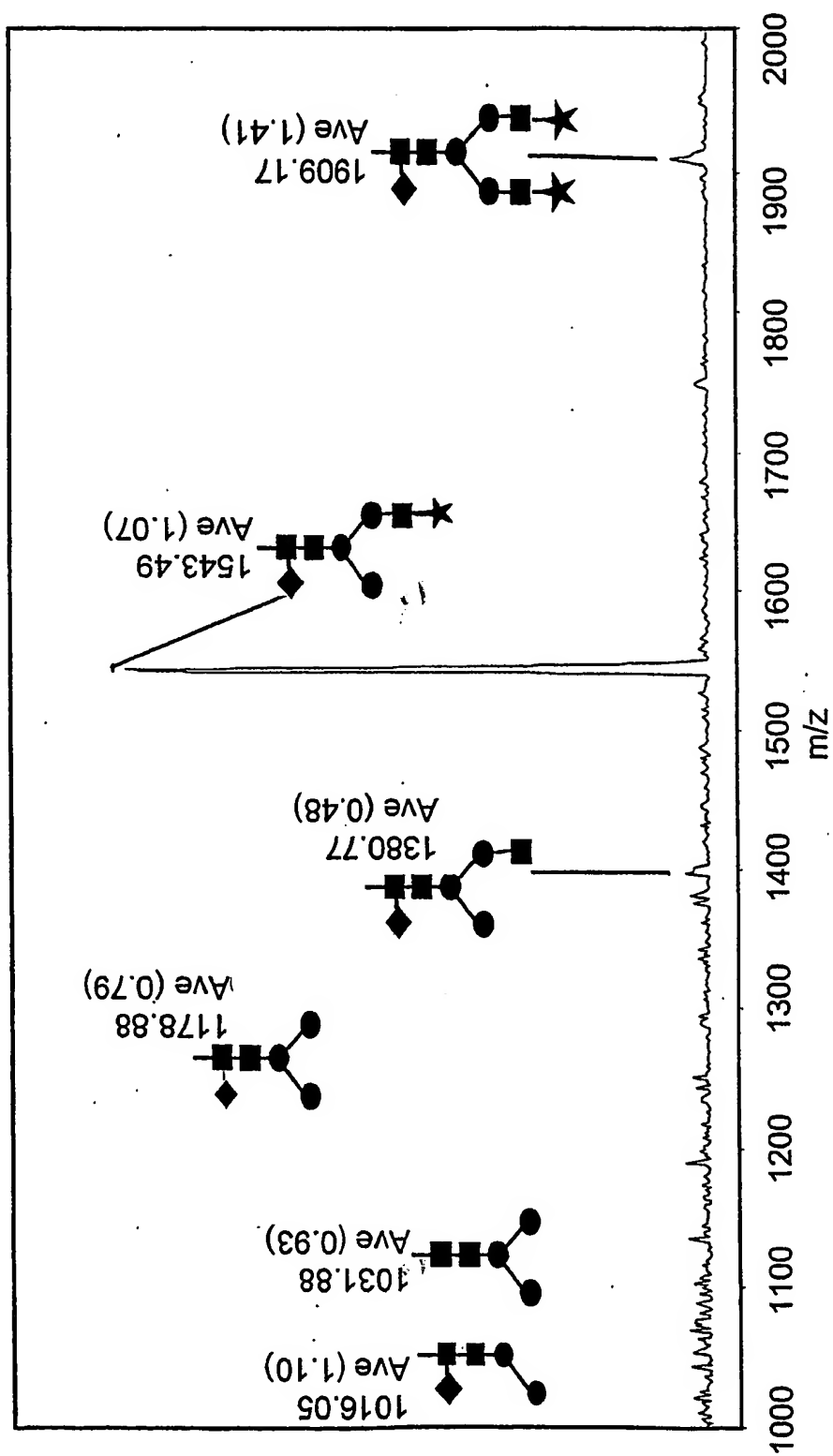


FIG. 140

440/498

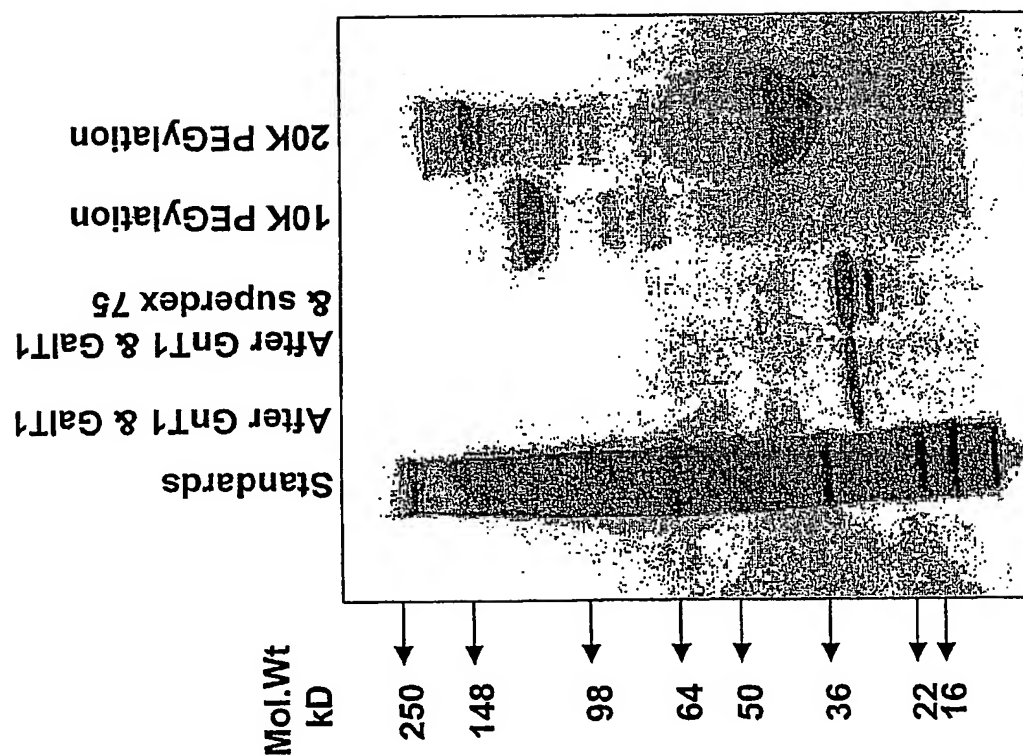


FIG. 141

441/498

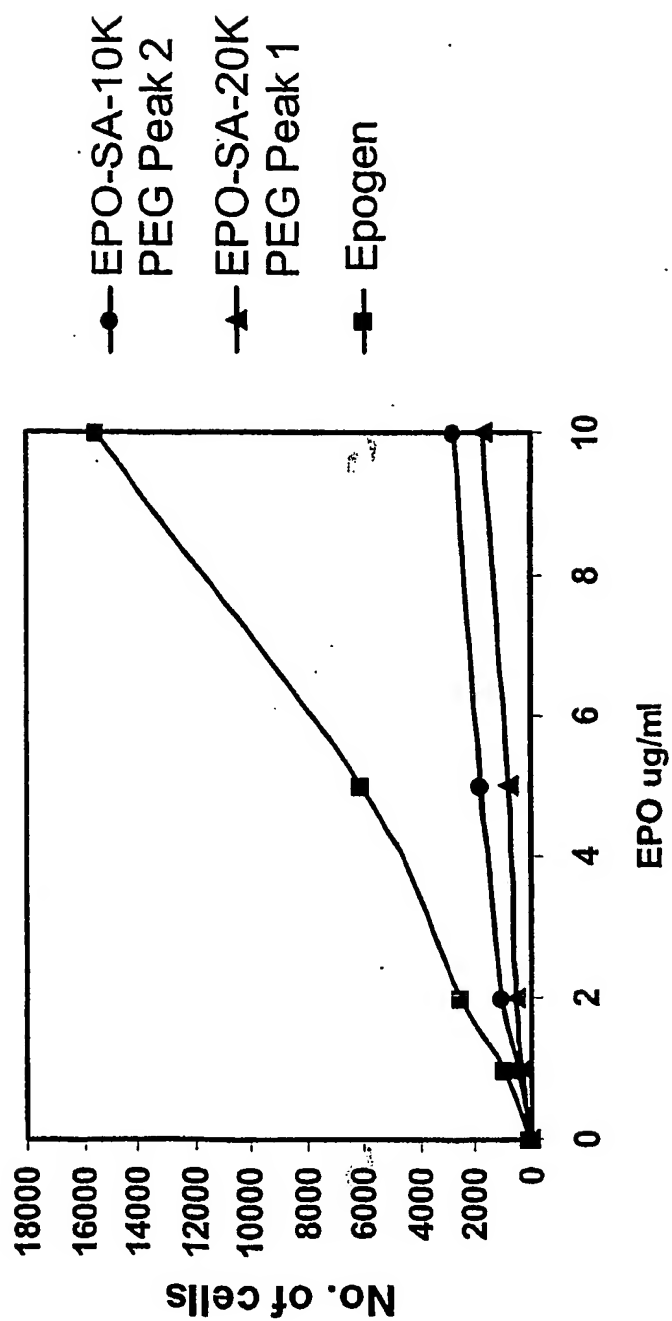


FIG. 142



442/498

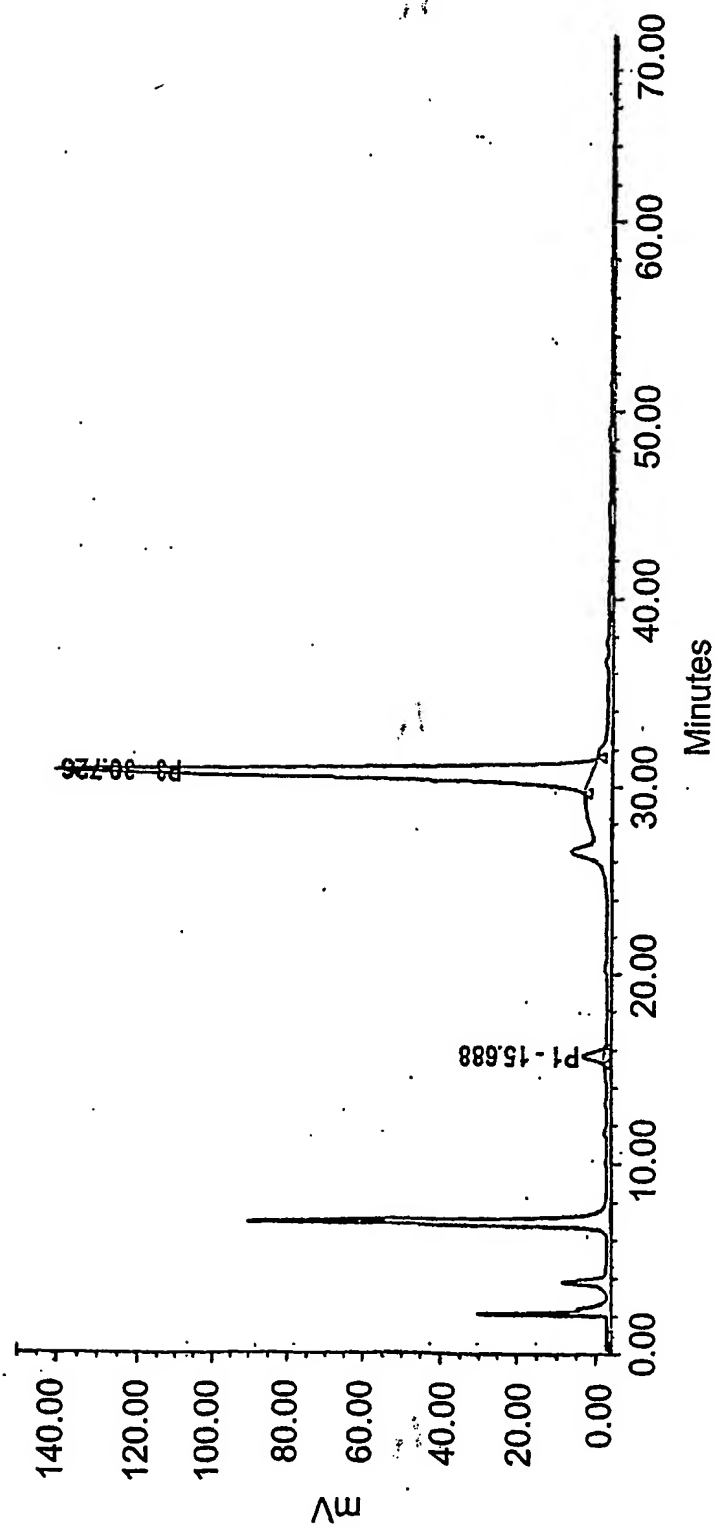


FIG. 143A

443/498

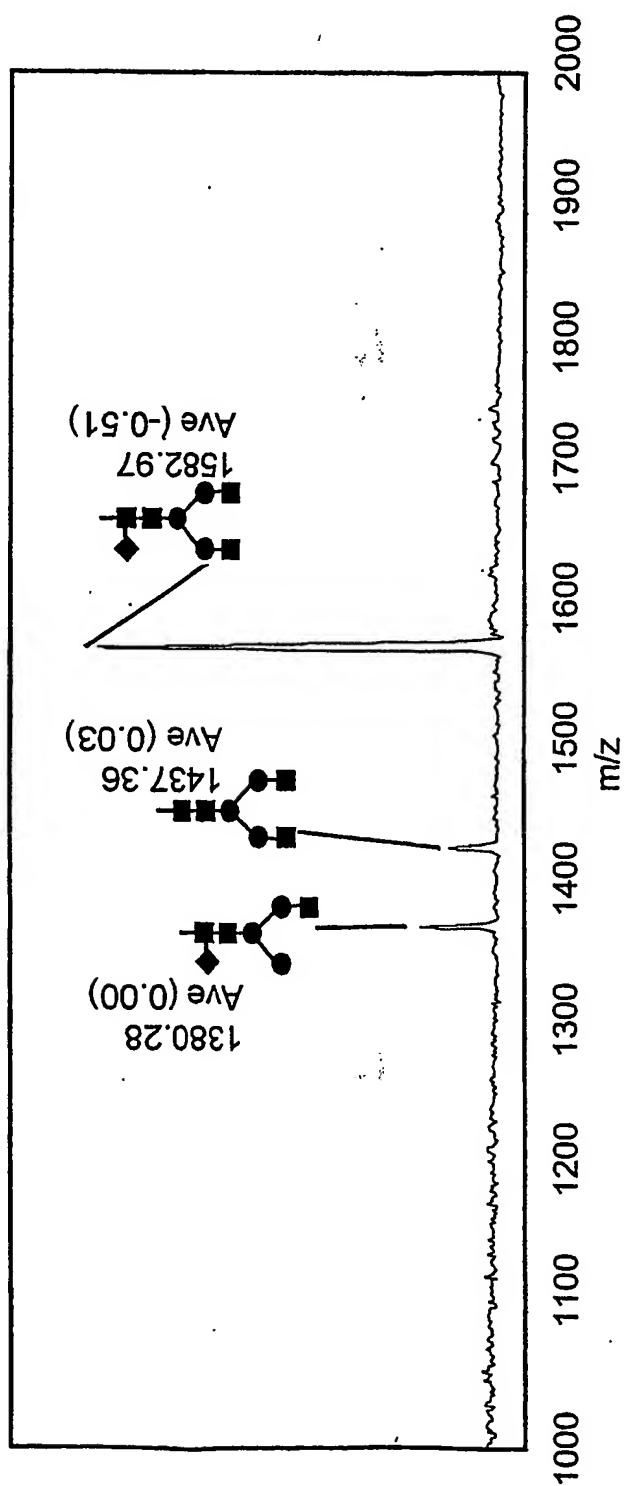


FIG. 143B

444/498

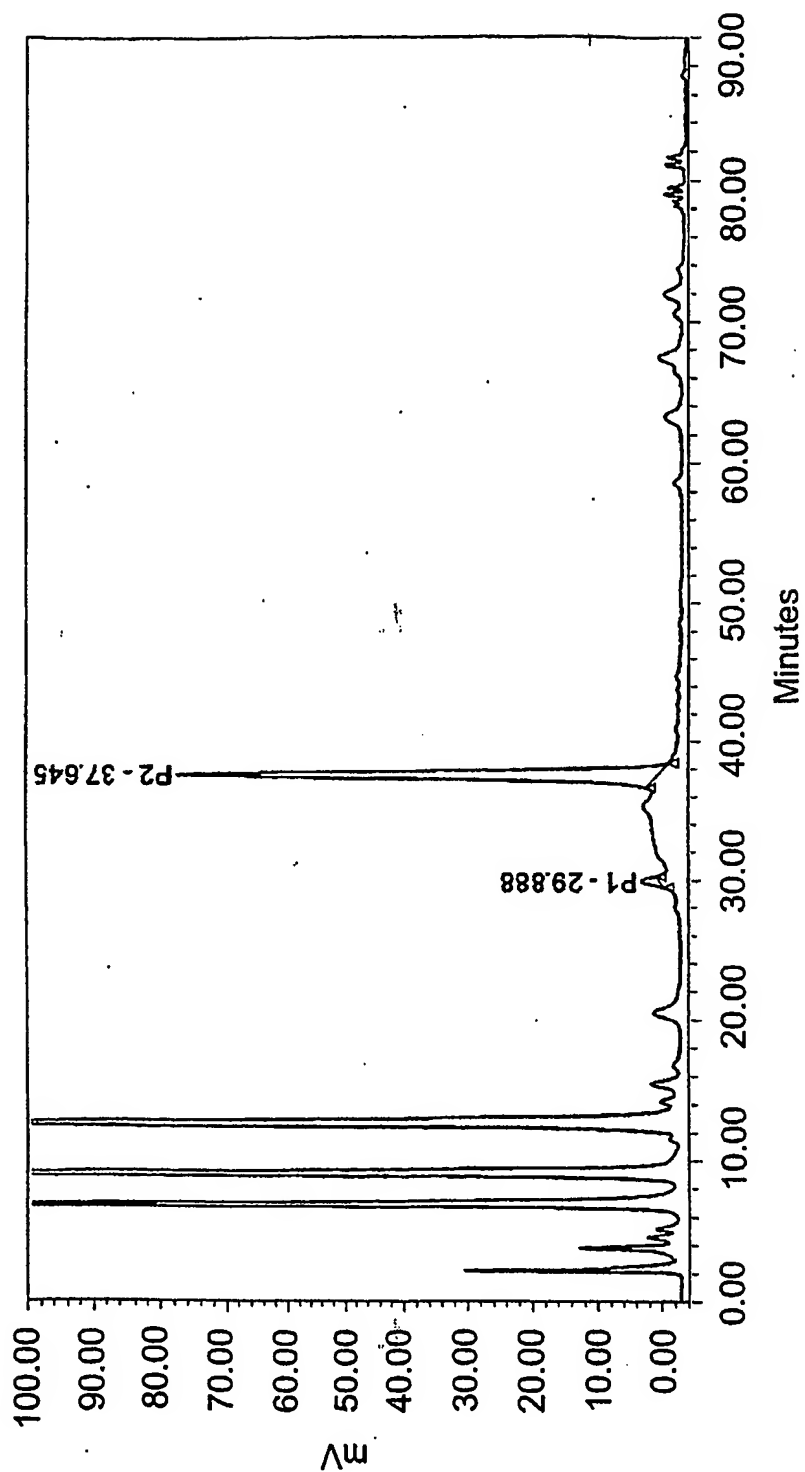


FIG. 144A

445/498

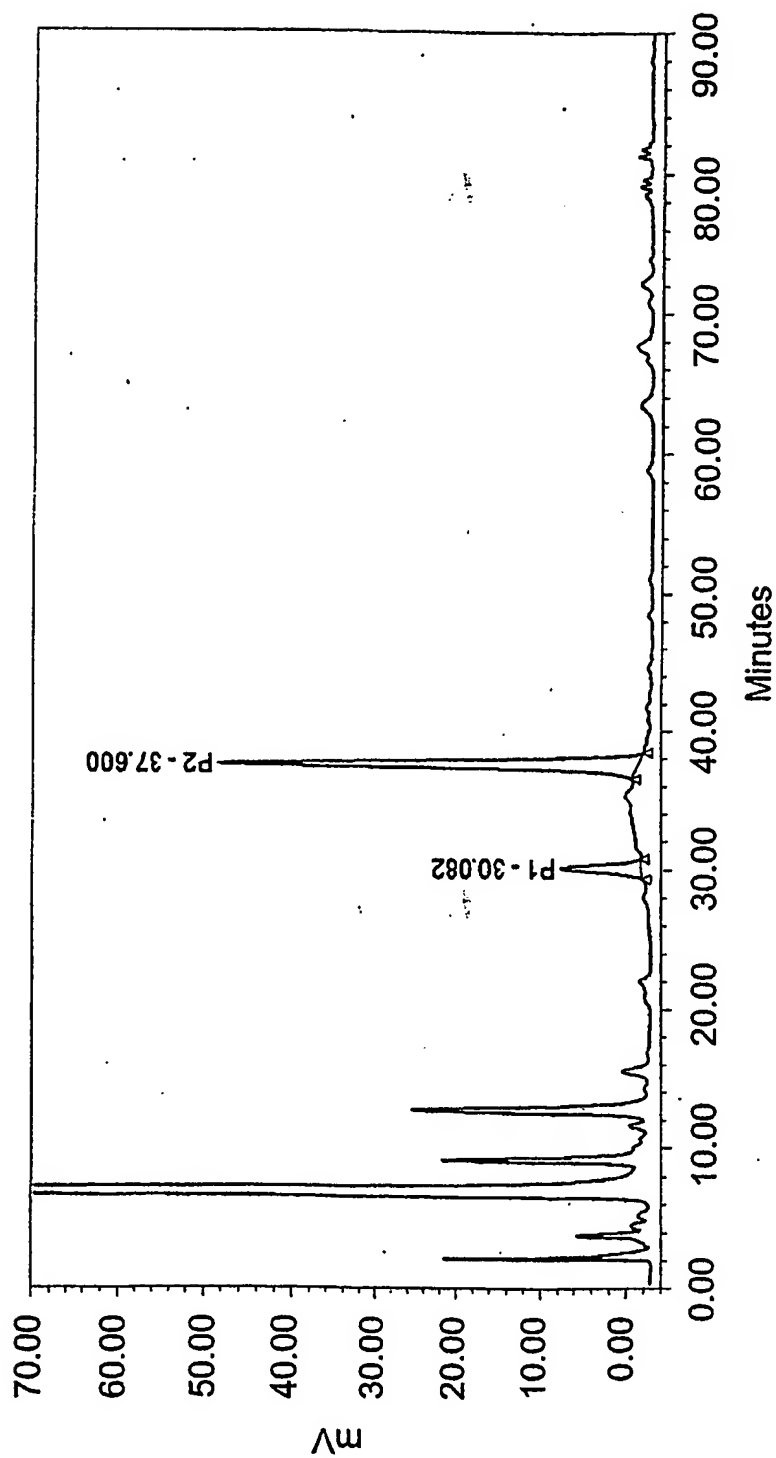


FIG. 144B

446/498

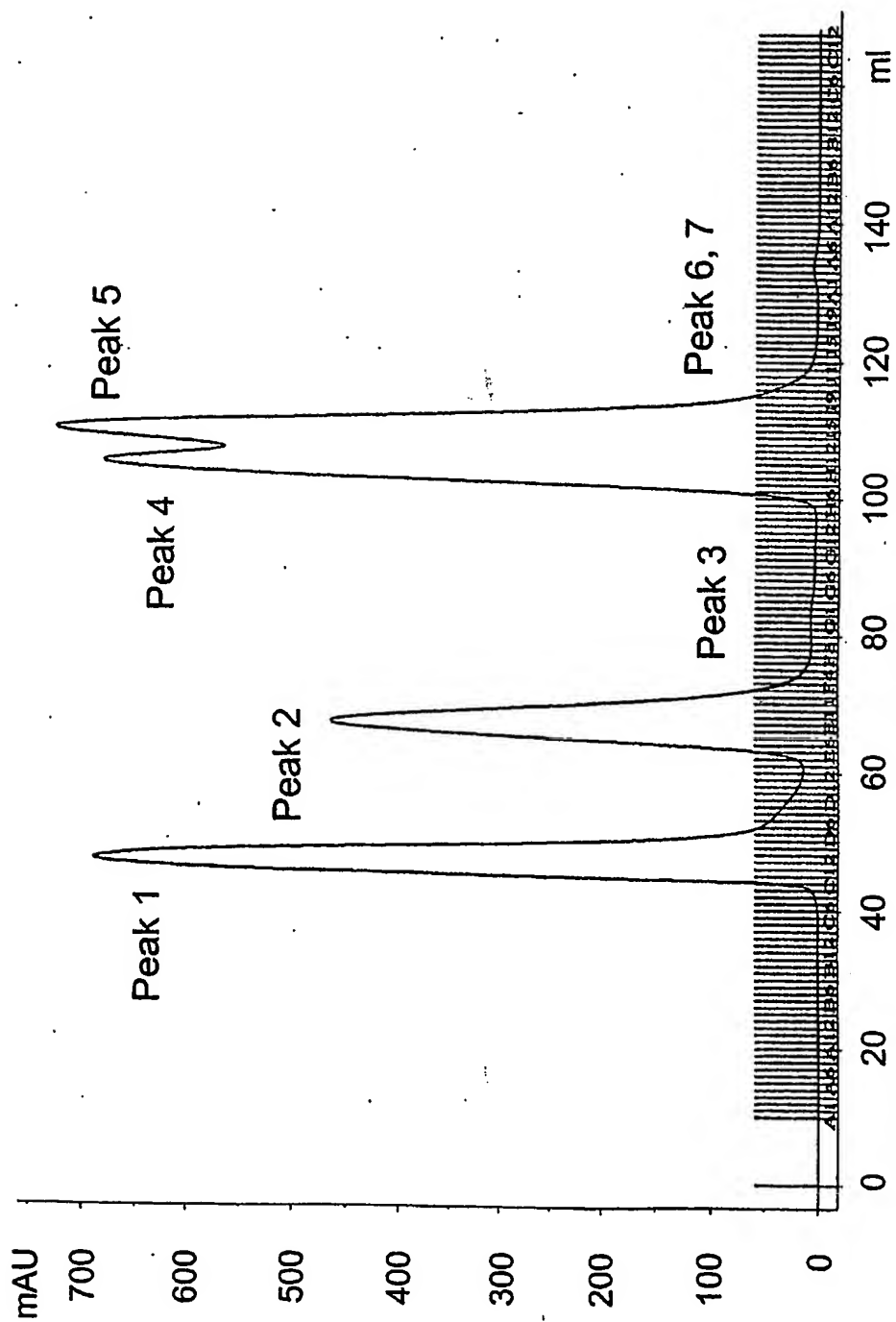


FIG. 145

447/498

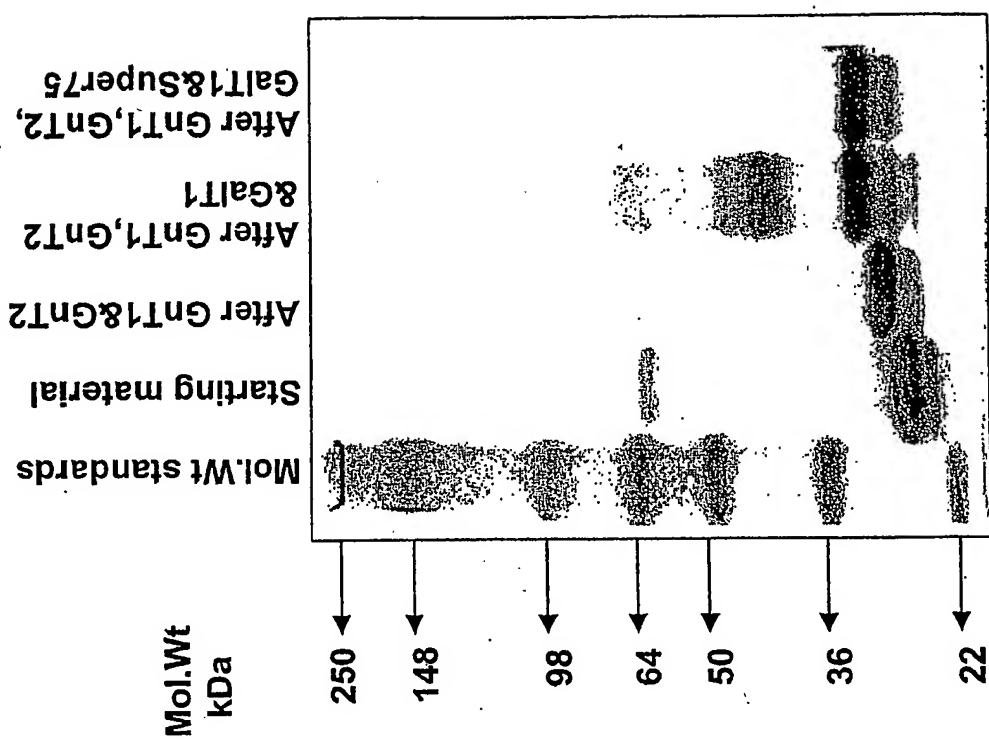


FIG. 146

448/498

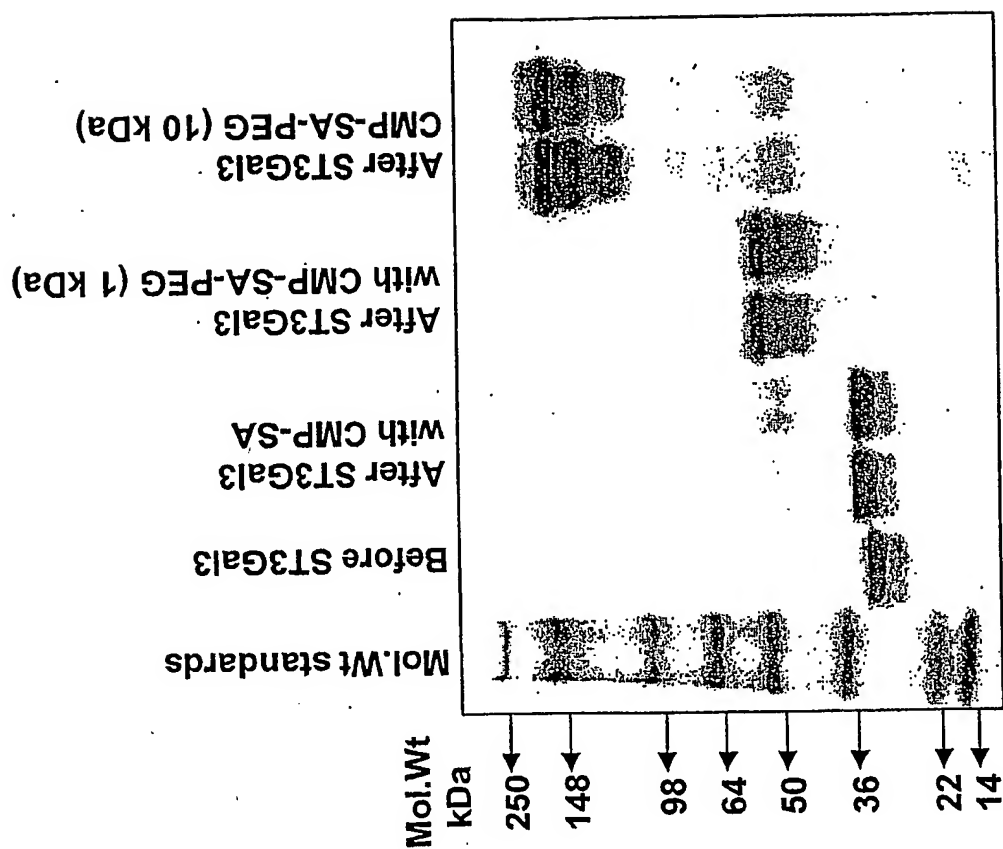


FIG. 147

449/498

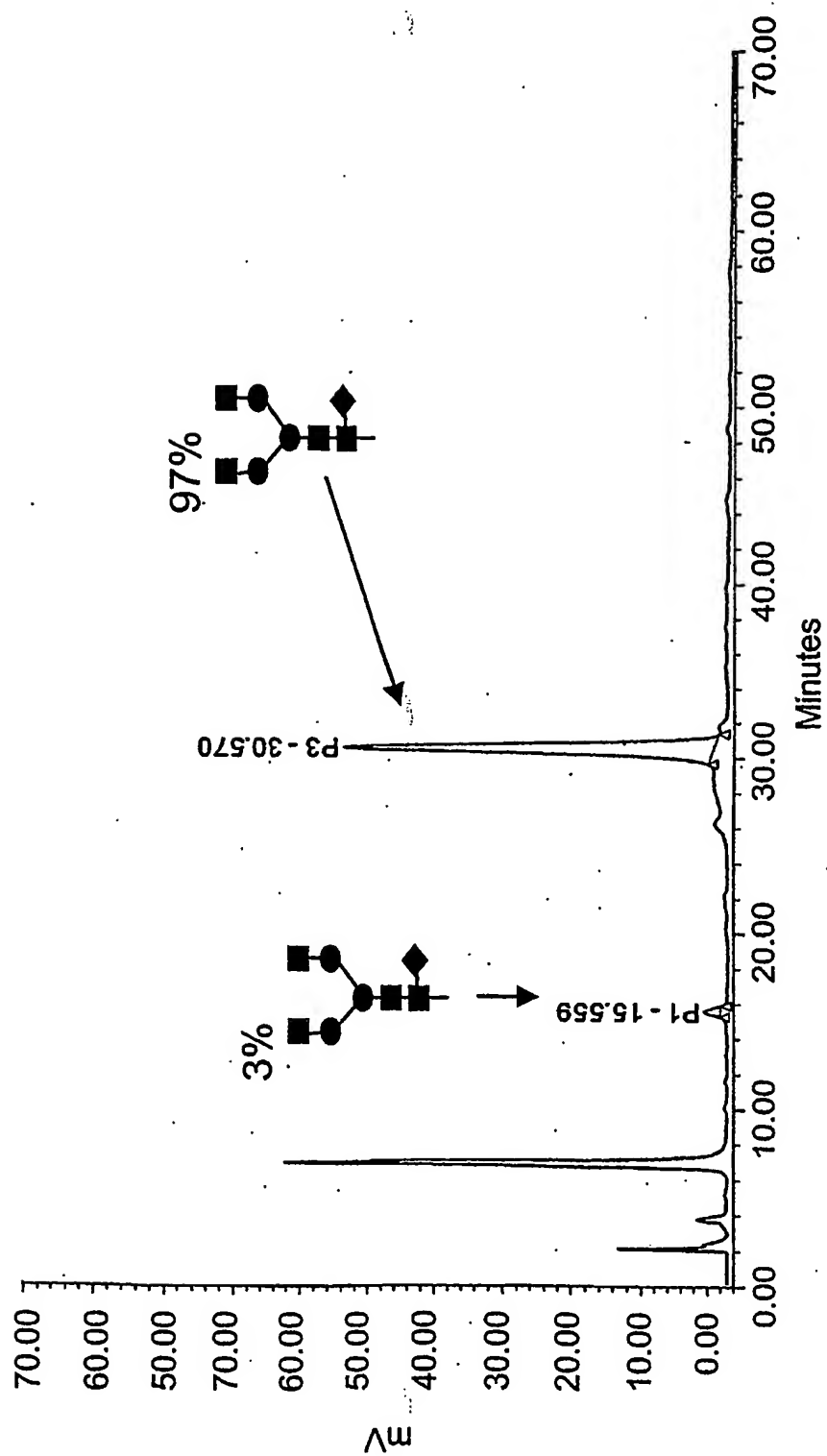


FIG. 148



450/498

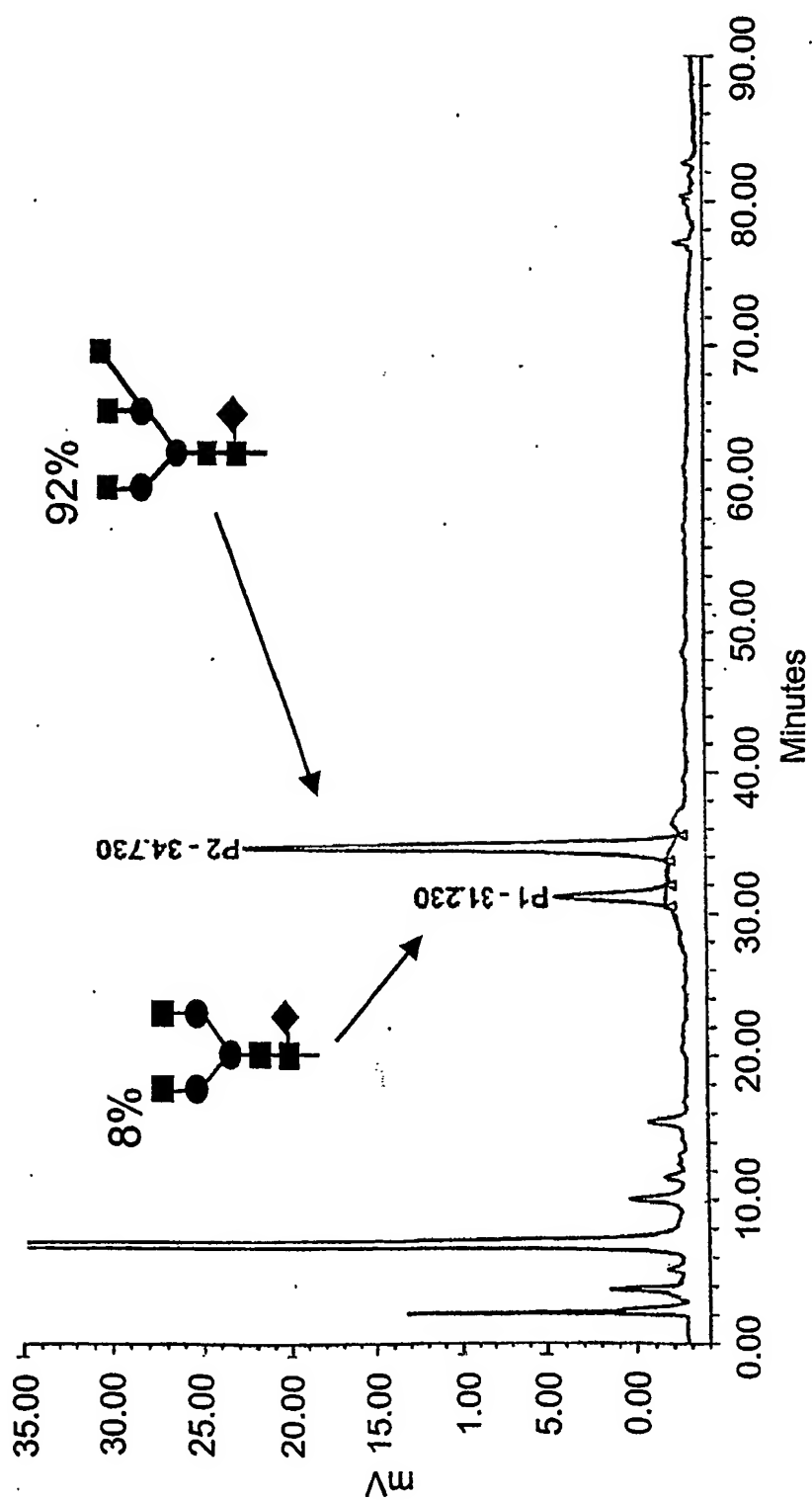


FIG. 149

451/498

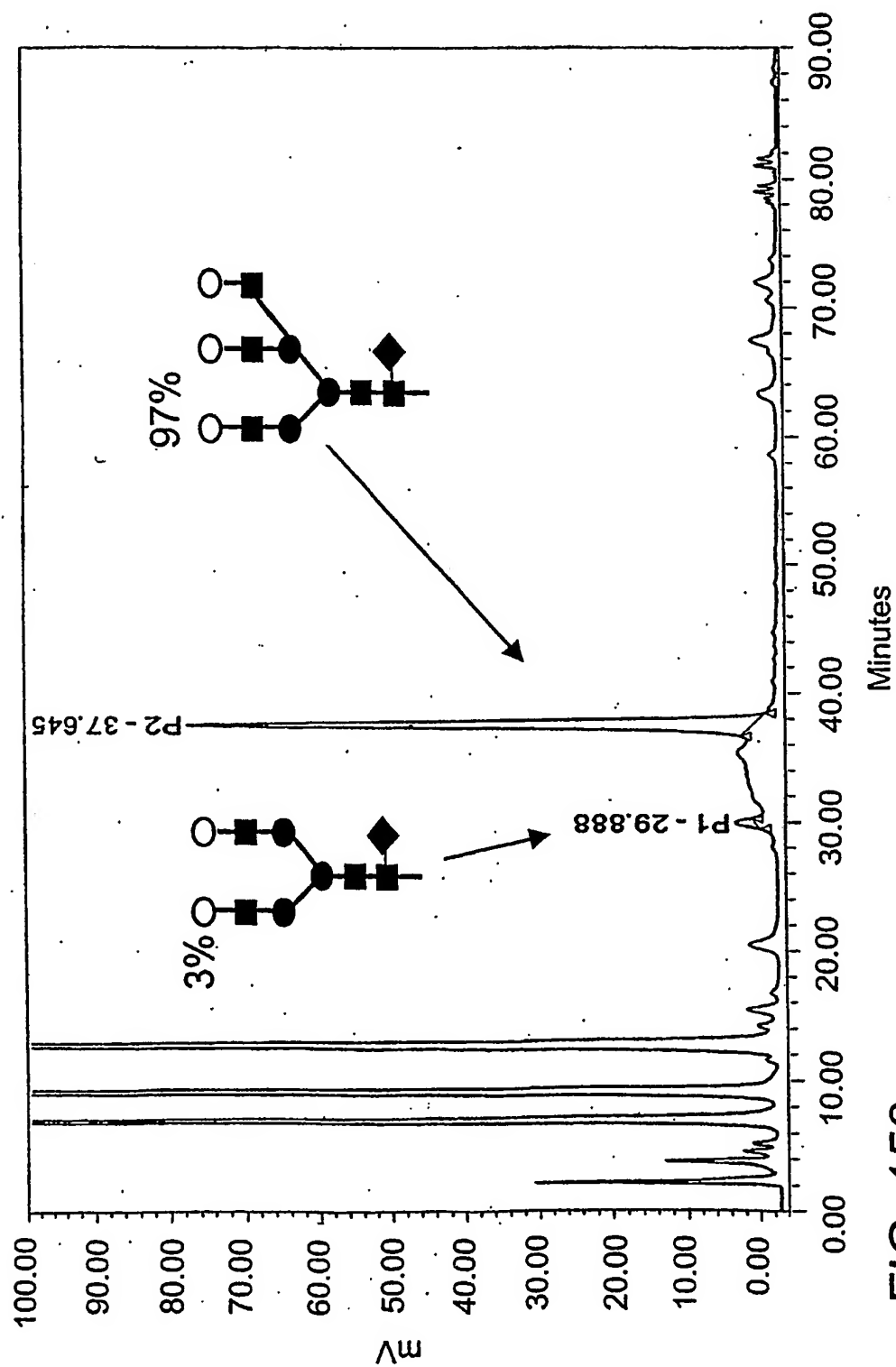


FIG. 150

452/498

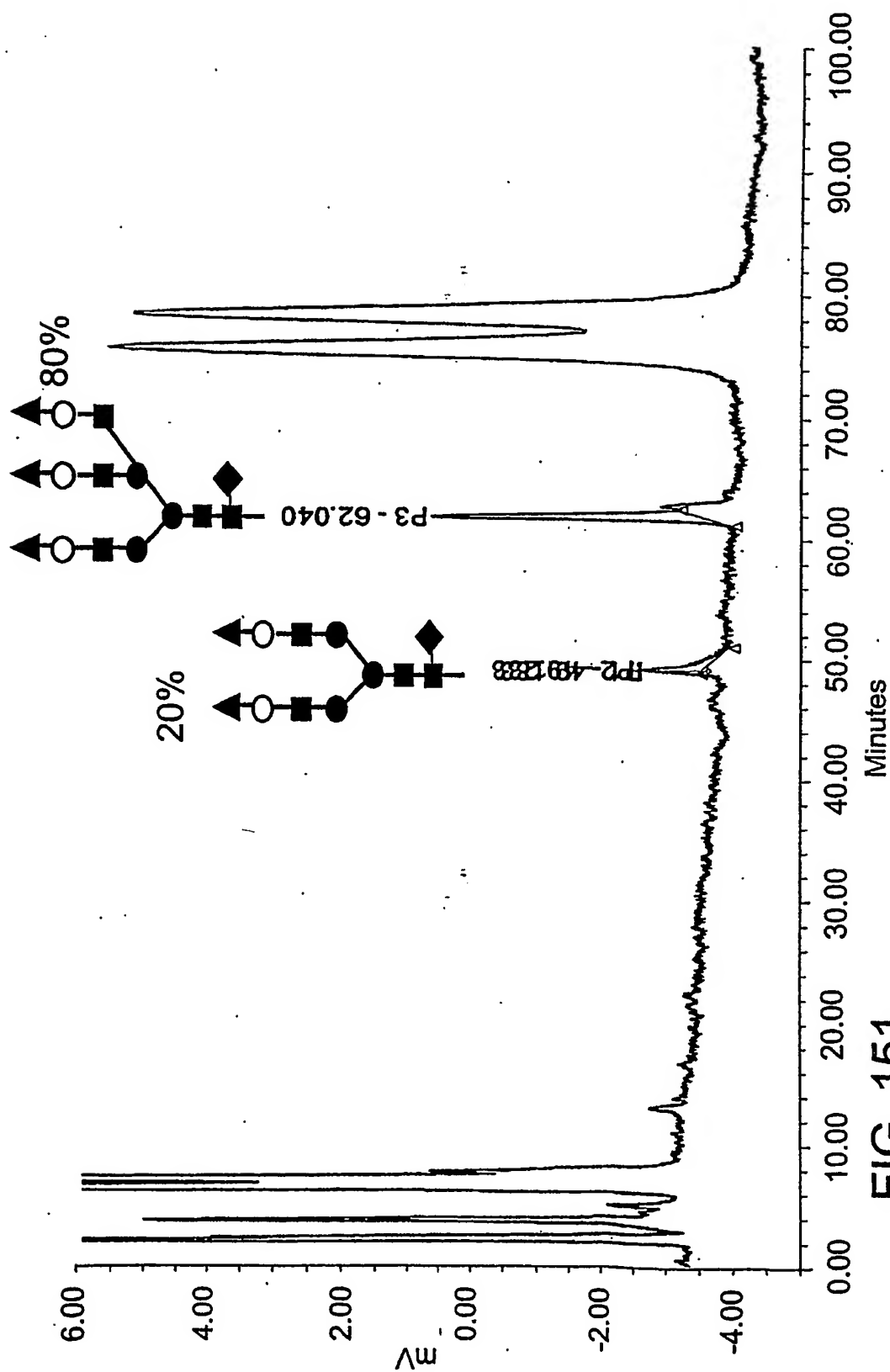


FIG. 151

453/498

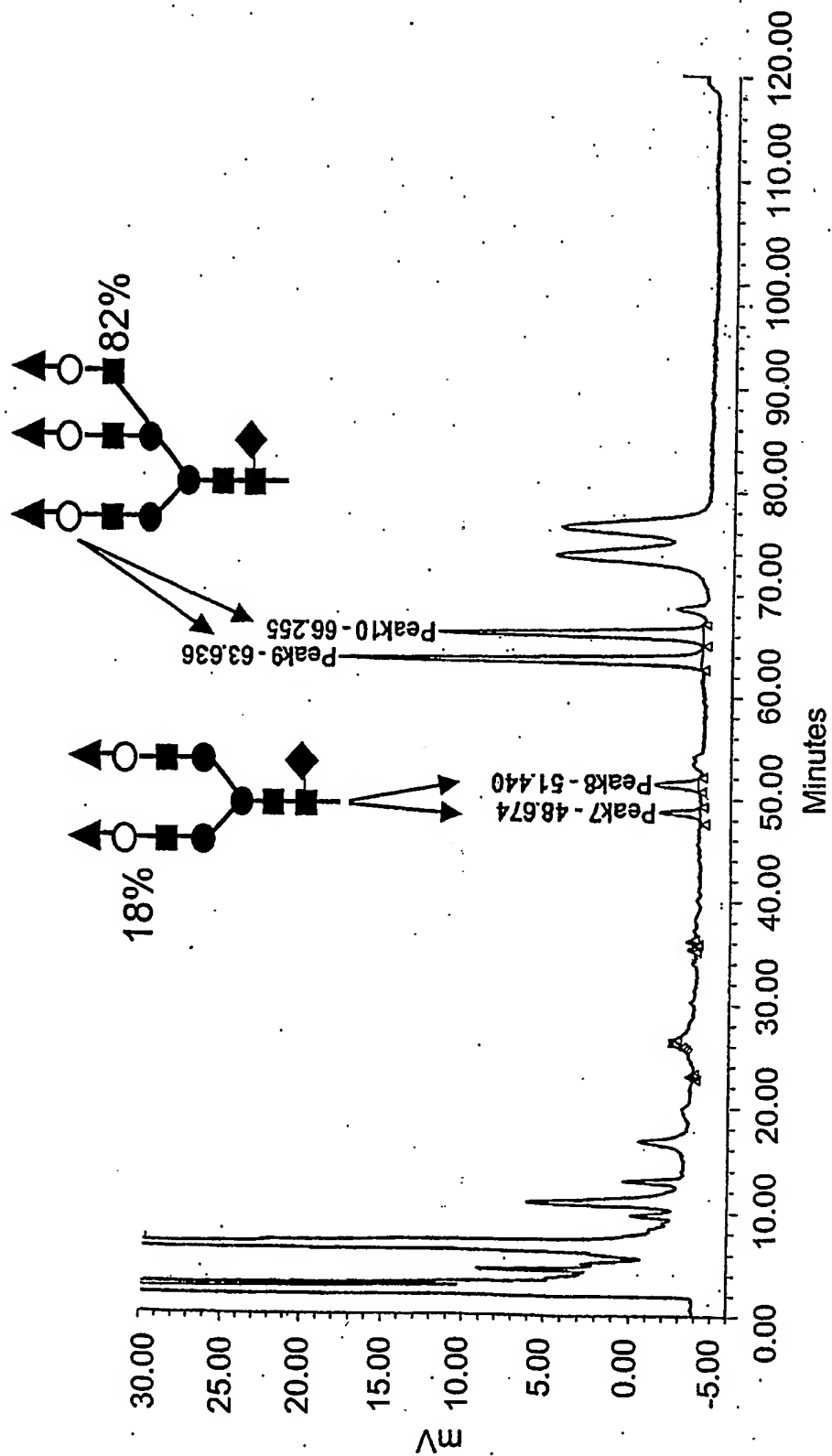
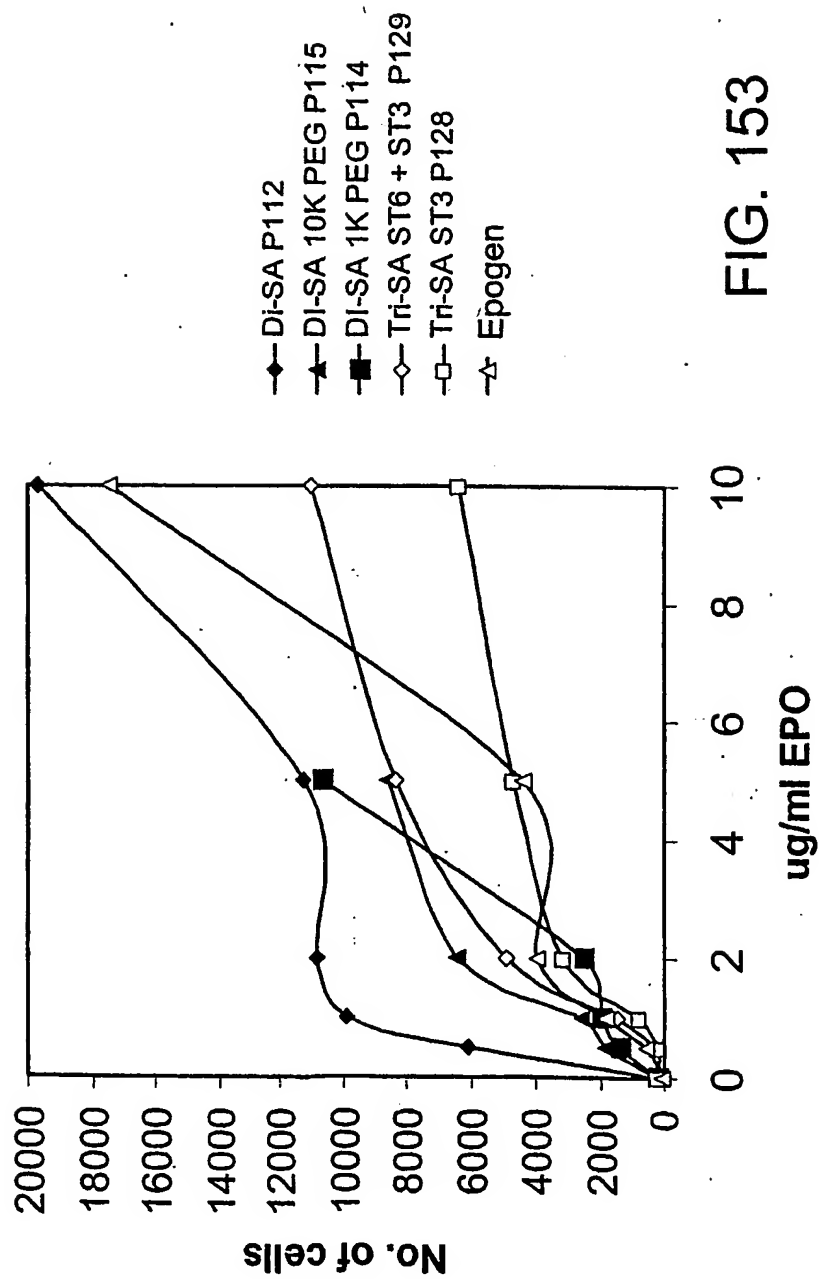


FIG. 152

454/498



455/498

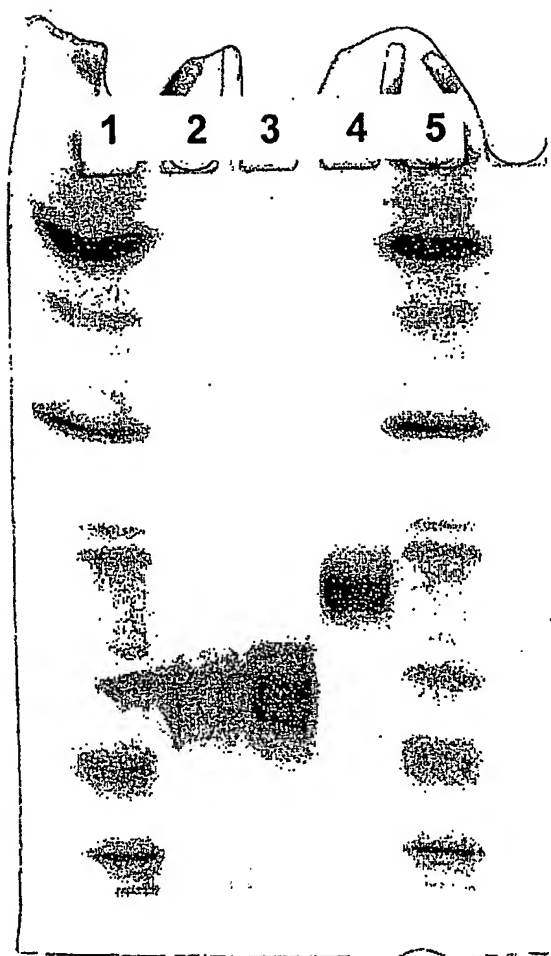


FIG. 154

456/498

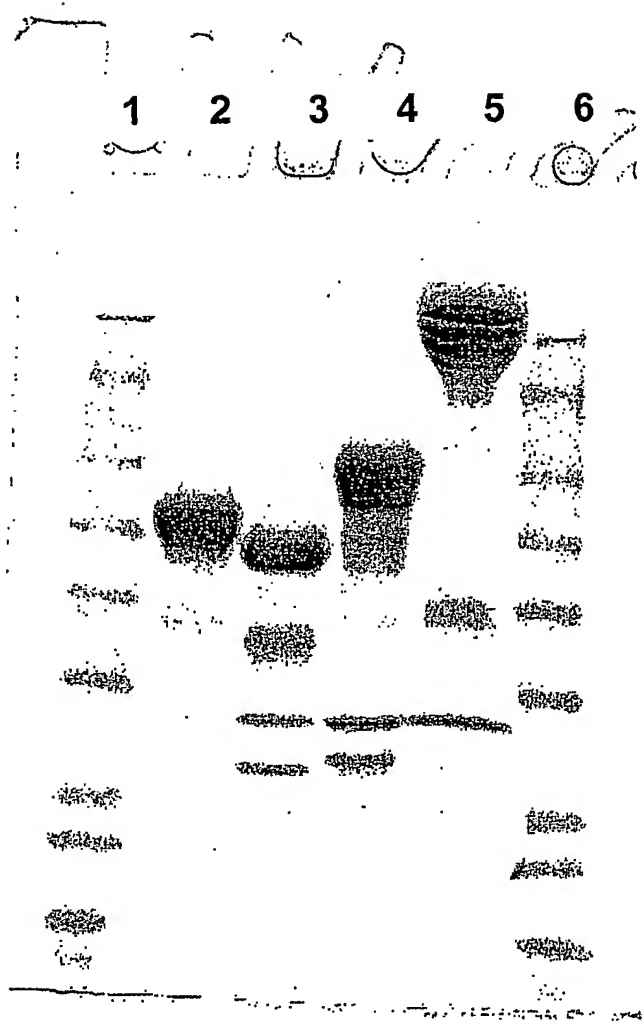


FIG. 155

457/498

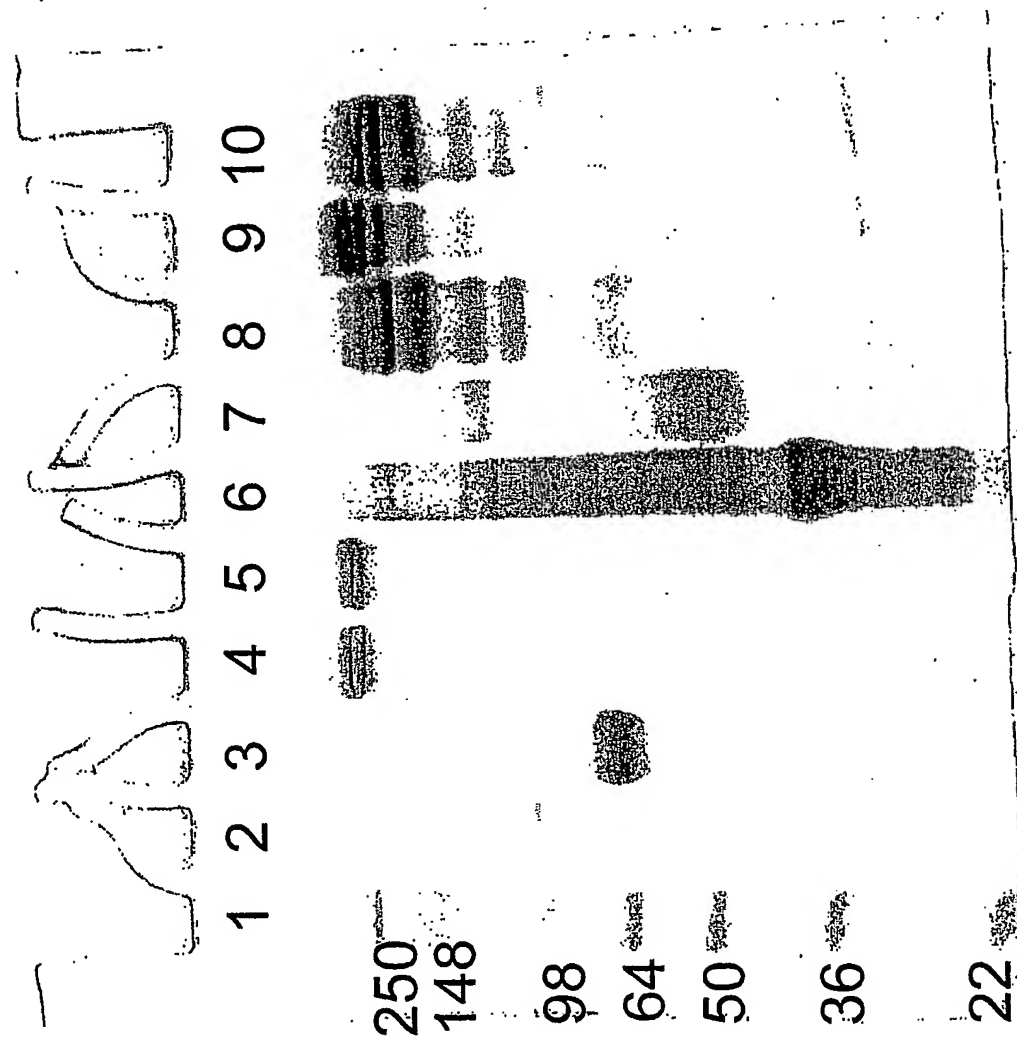


FIG. 156



458/498

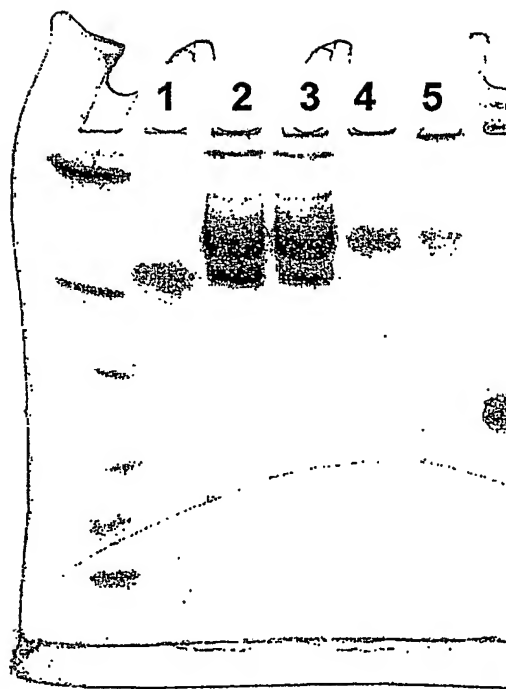


FIG. 157

459/498

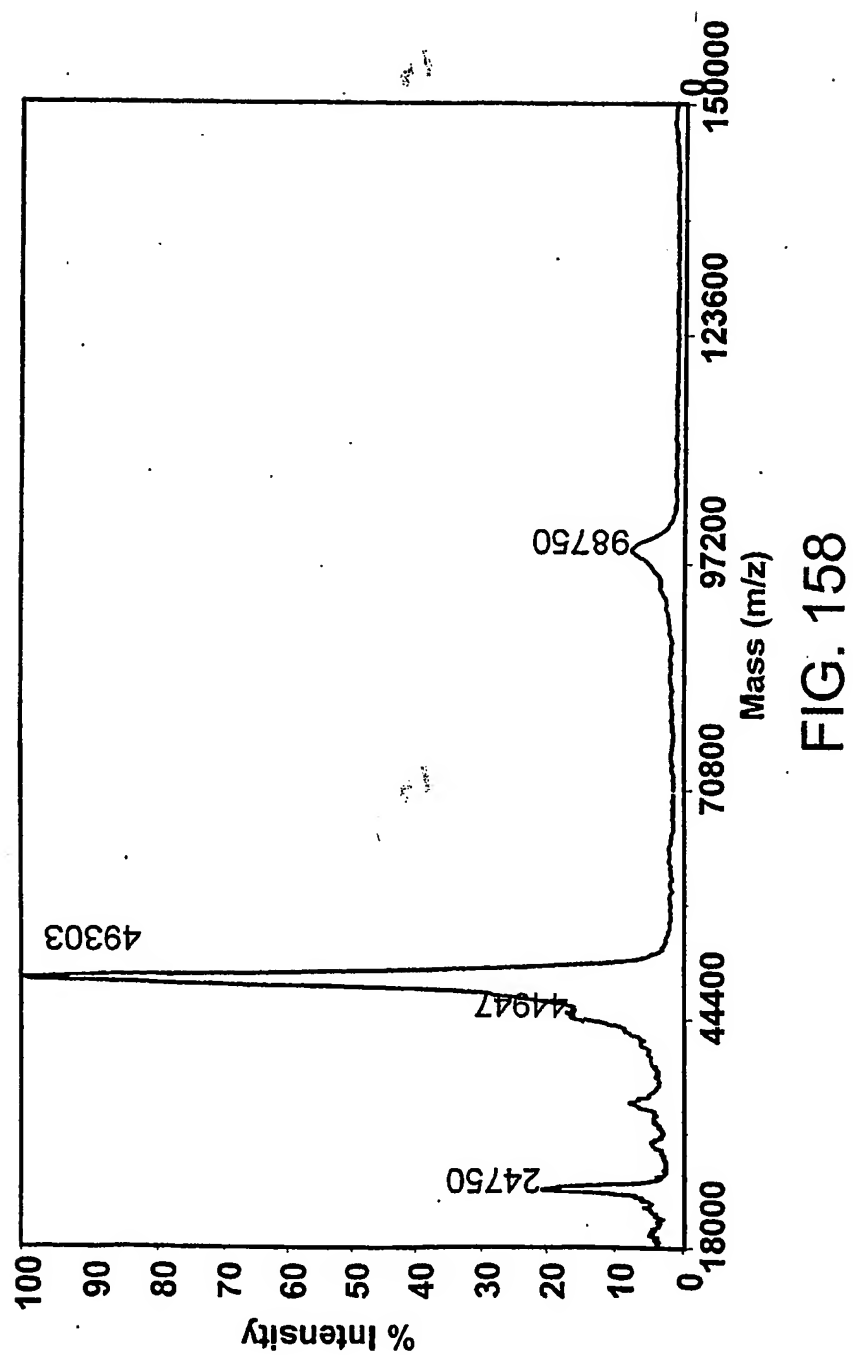


FIG. 158

460/498

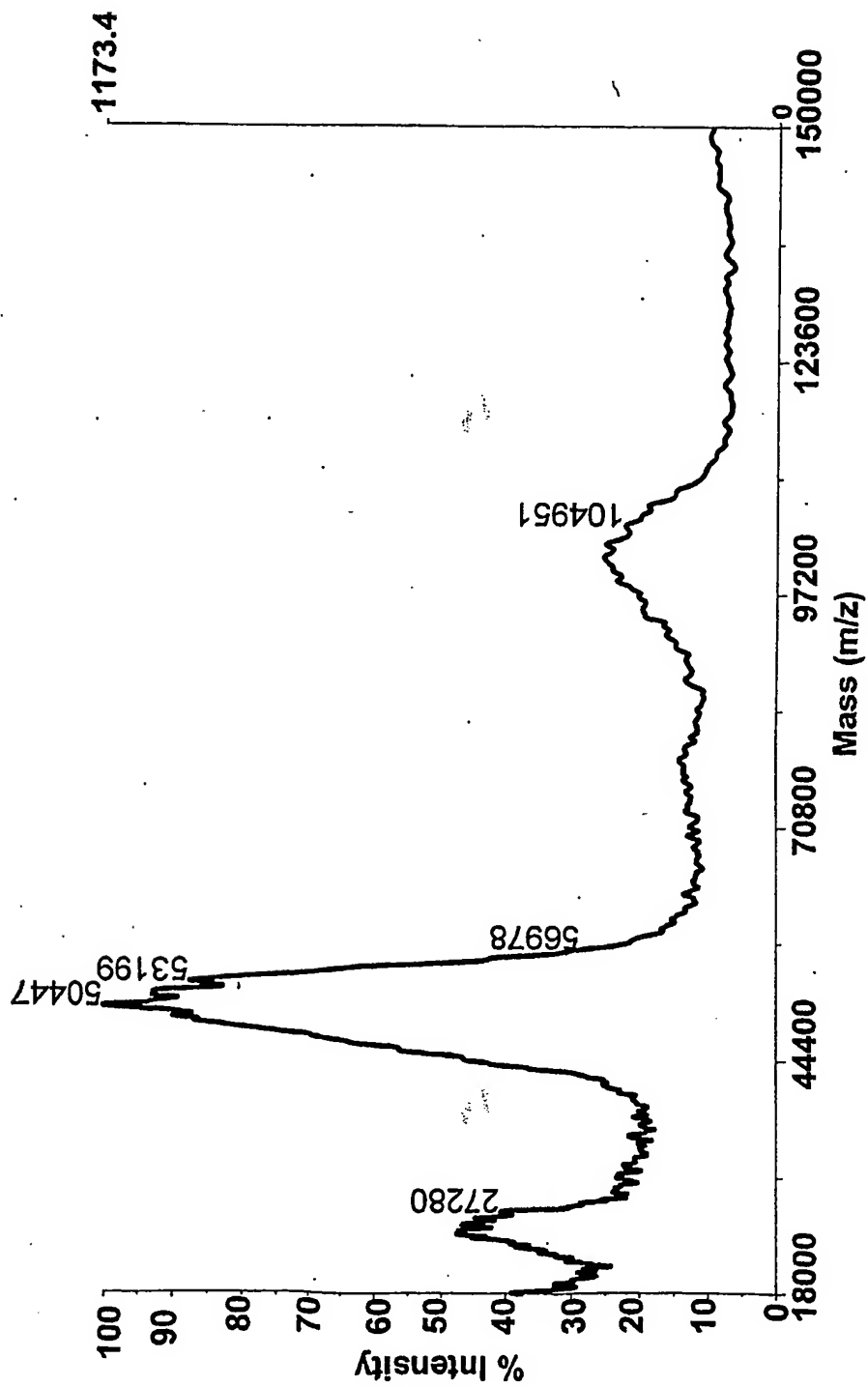


FIG. 159

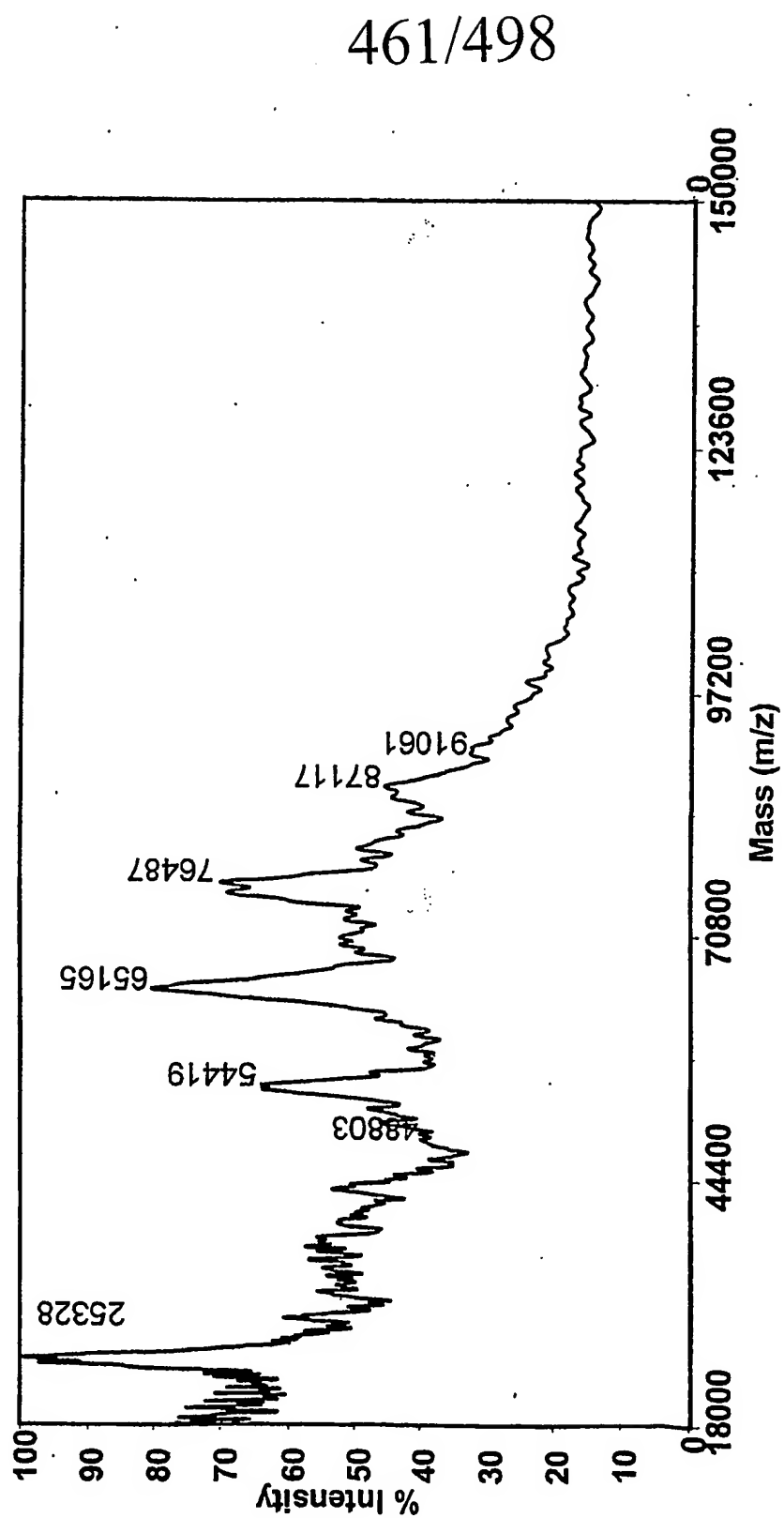


FIG. 160

462/498

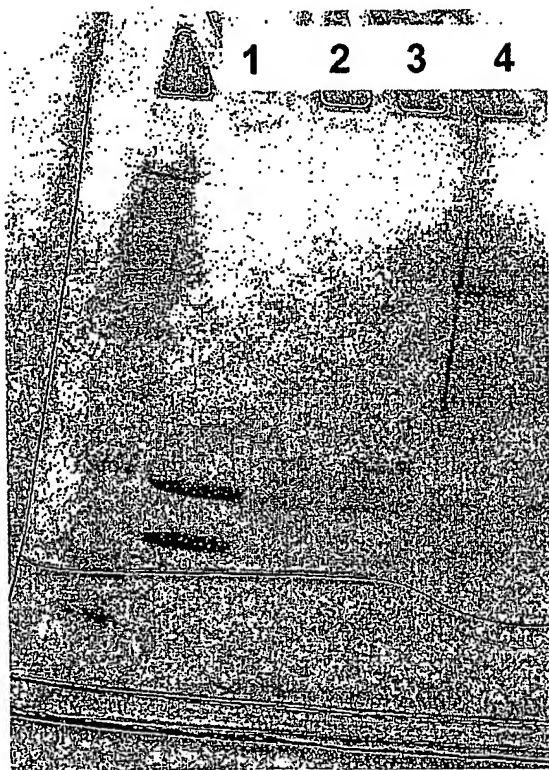


FIG. 161

463/498

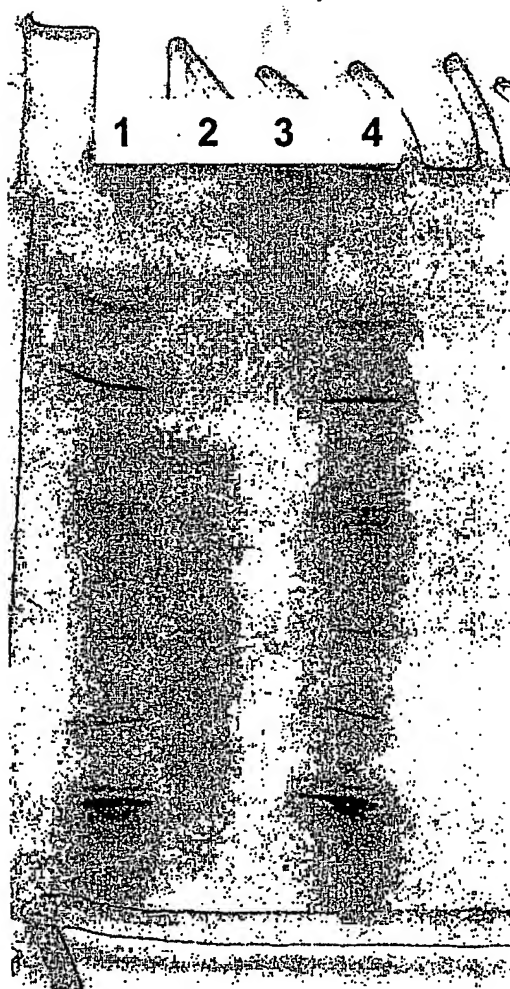


FIG. 162

464/498

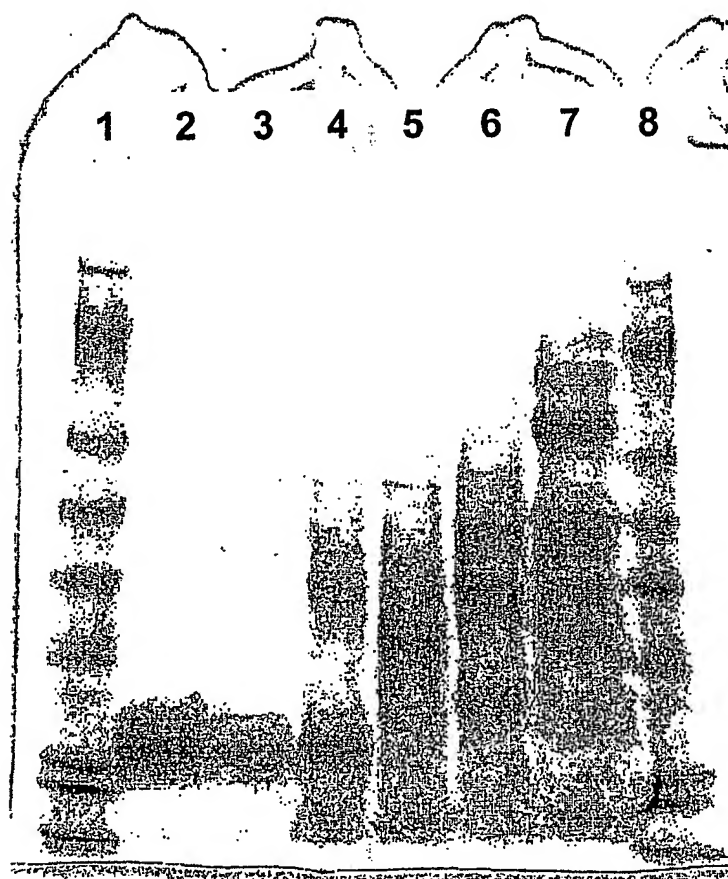


FIG. 163

465/498

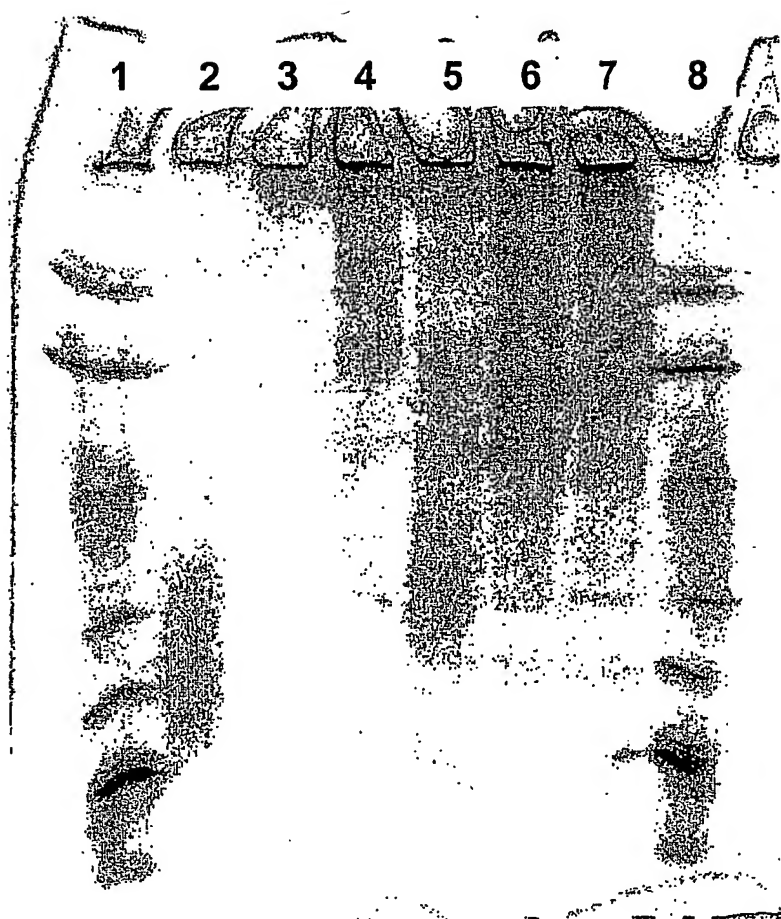


FIG. 164



466/498

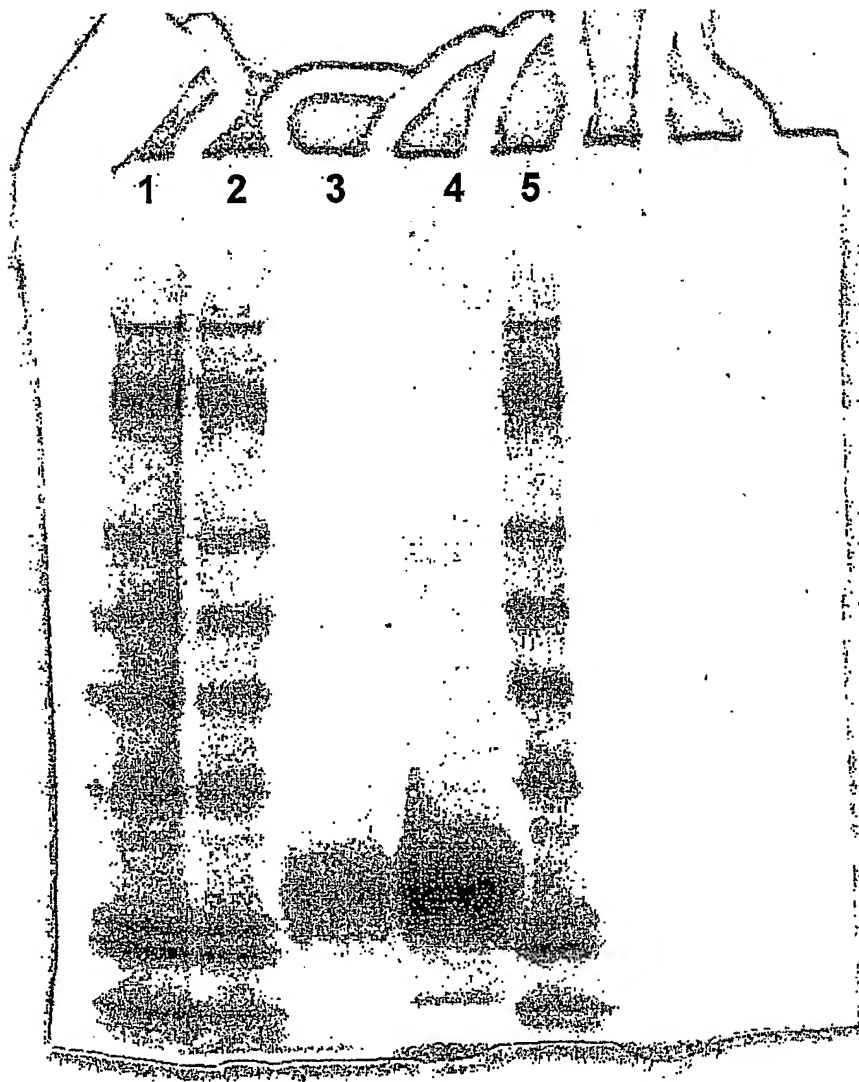


FIG. 165

467/498

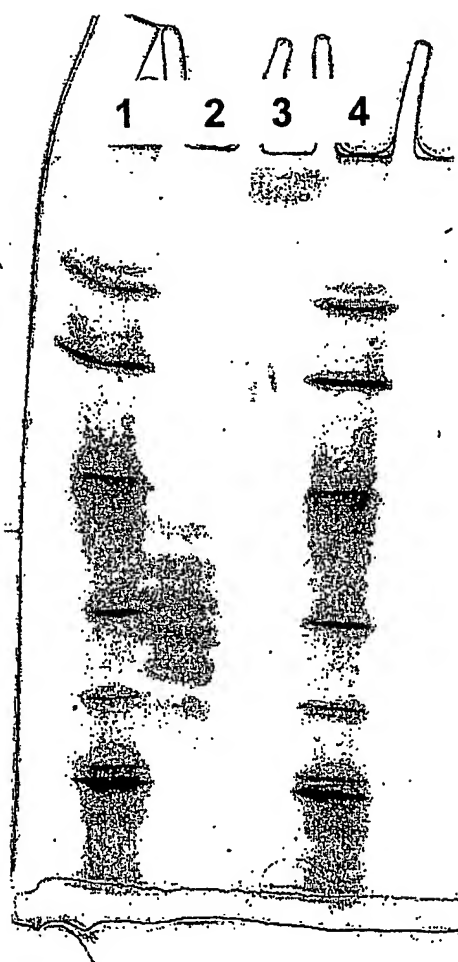


FIG. 166

468/498

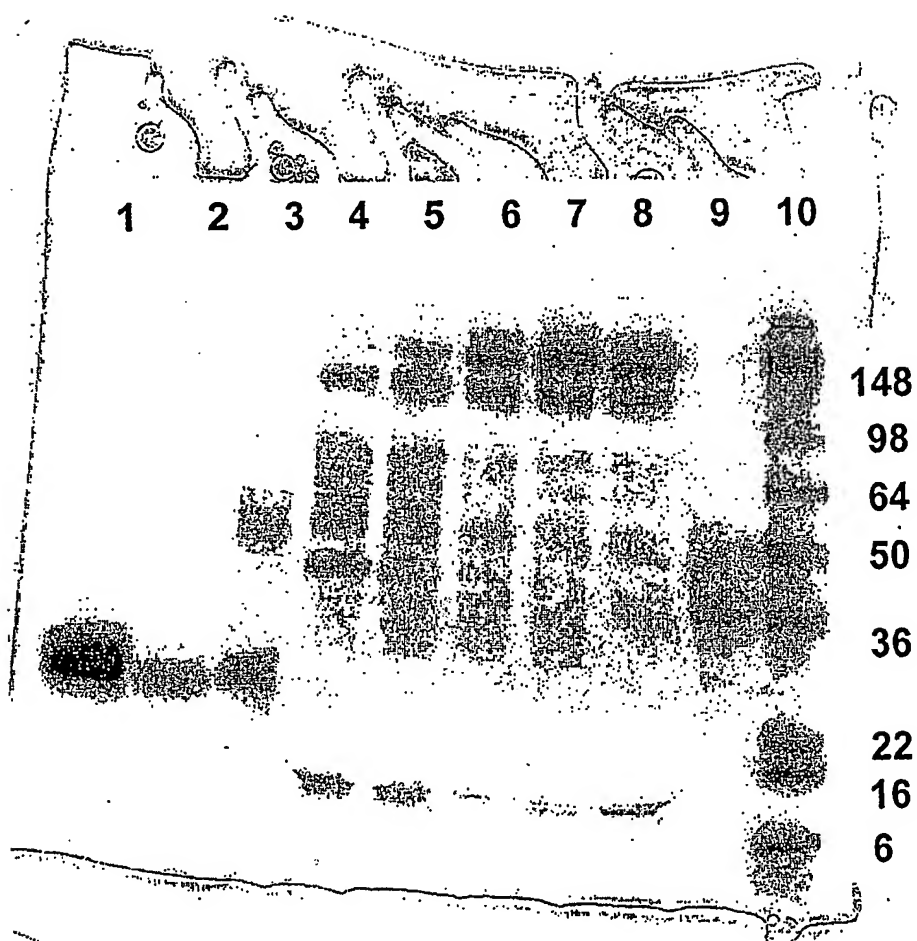


FIG. 167

469/498

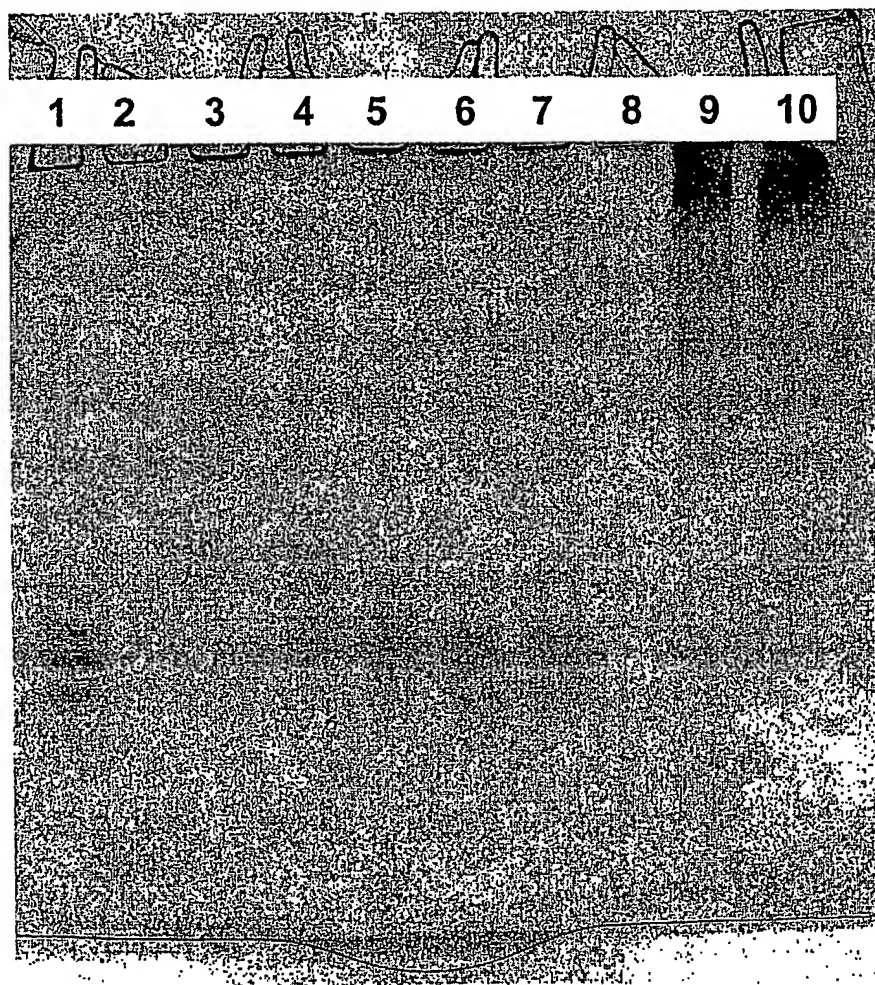


FIG. 168

470/498

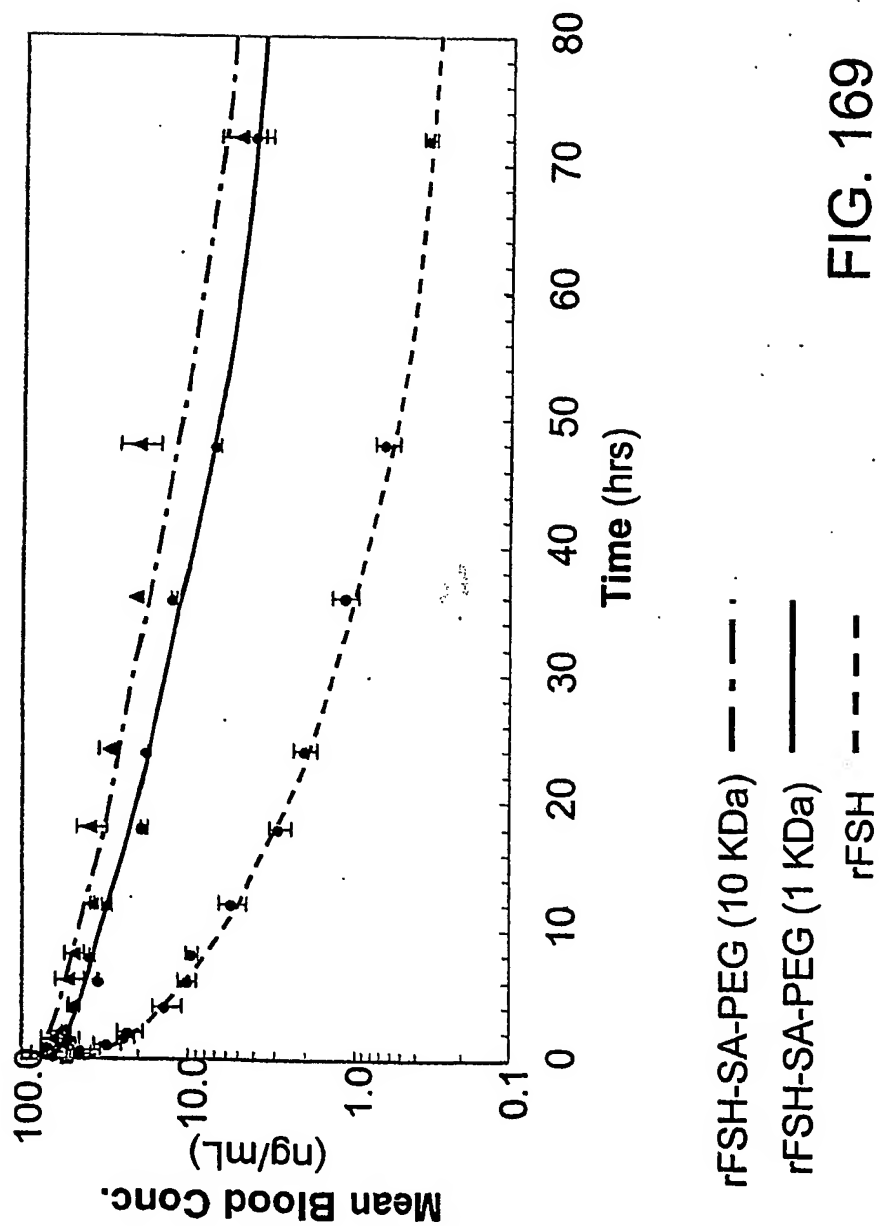


FIG. 169

471/498

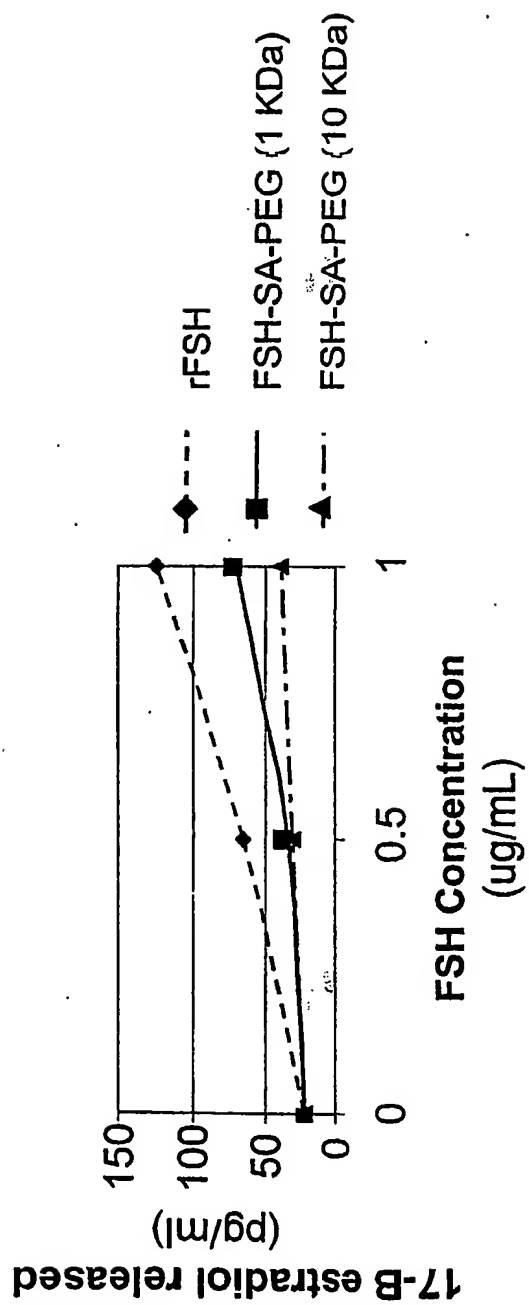


FIG. 170

472/498

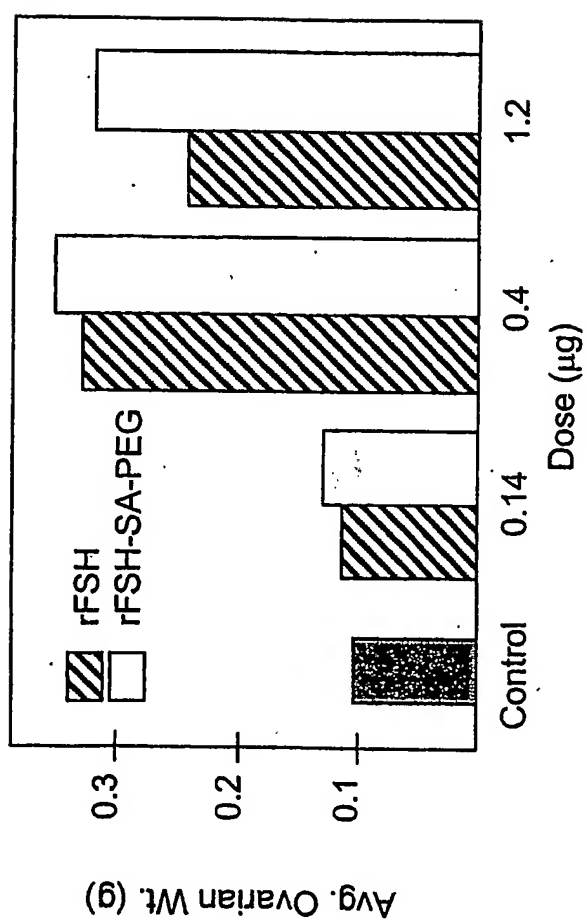


FIG. 171

473/498

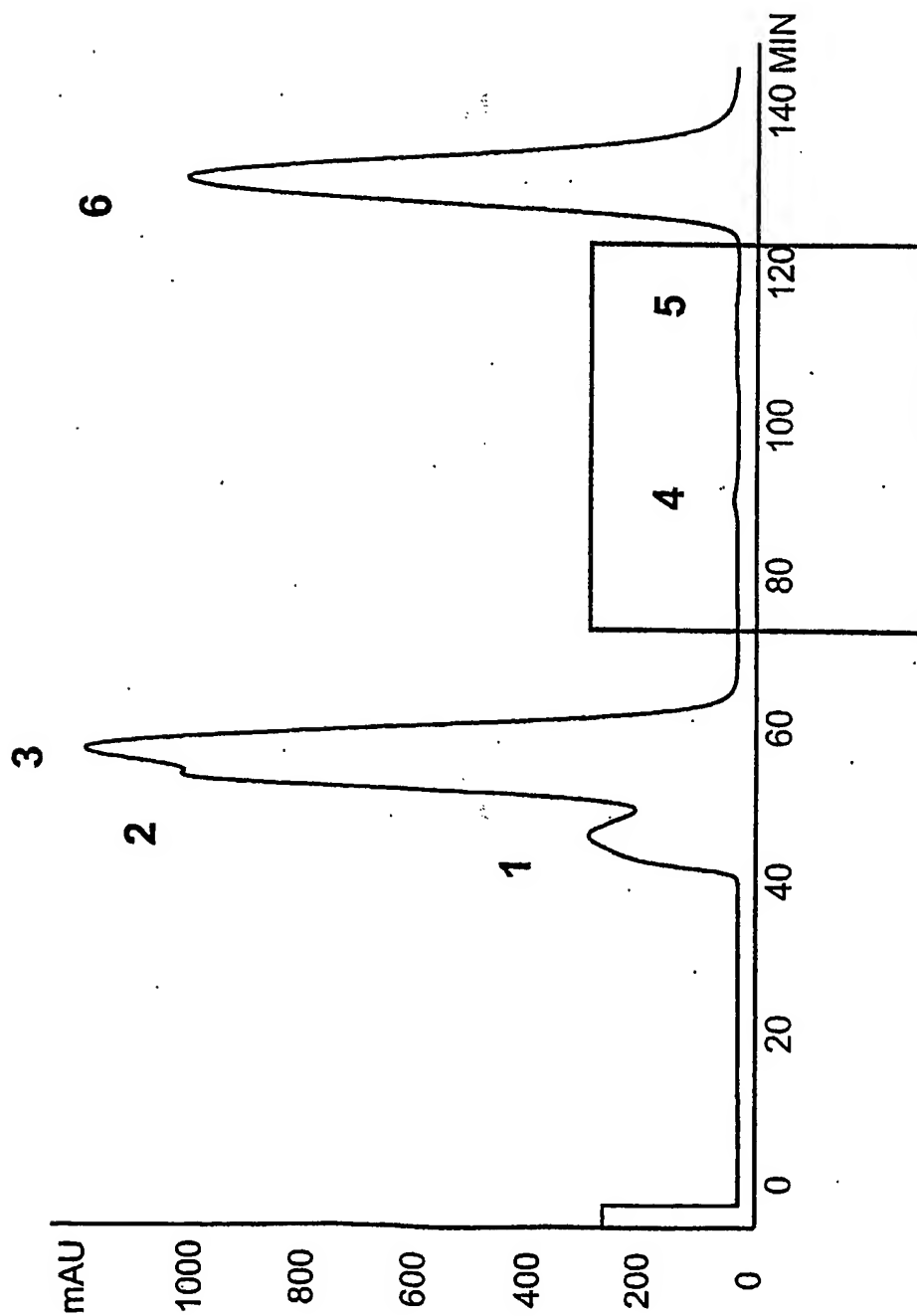


FIG. 172A



474/498

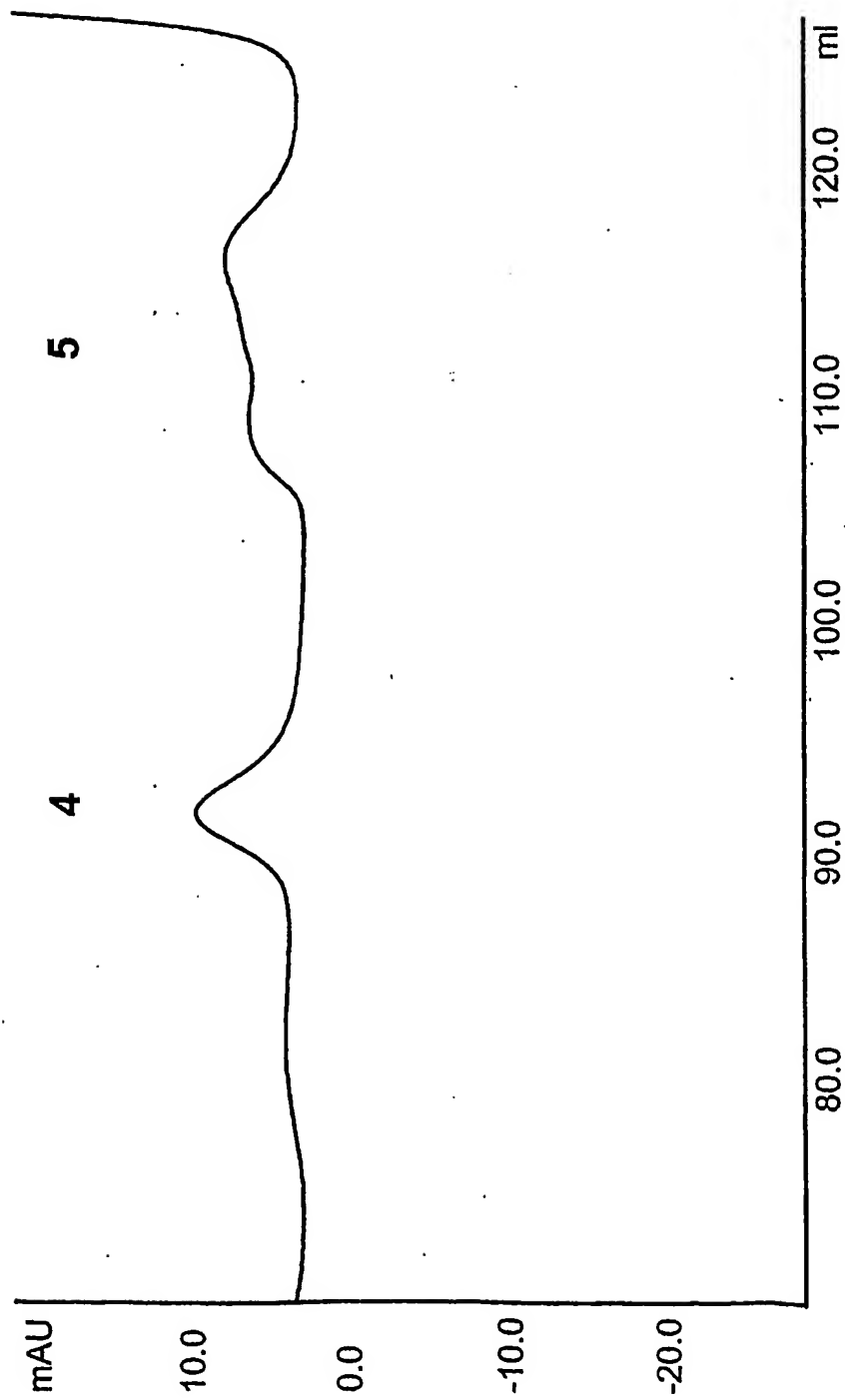


FIG. 172B

475/498

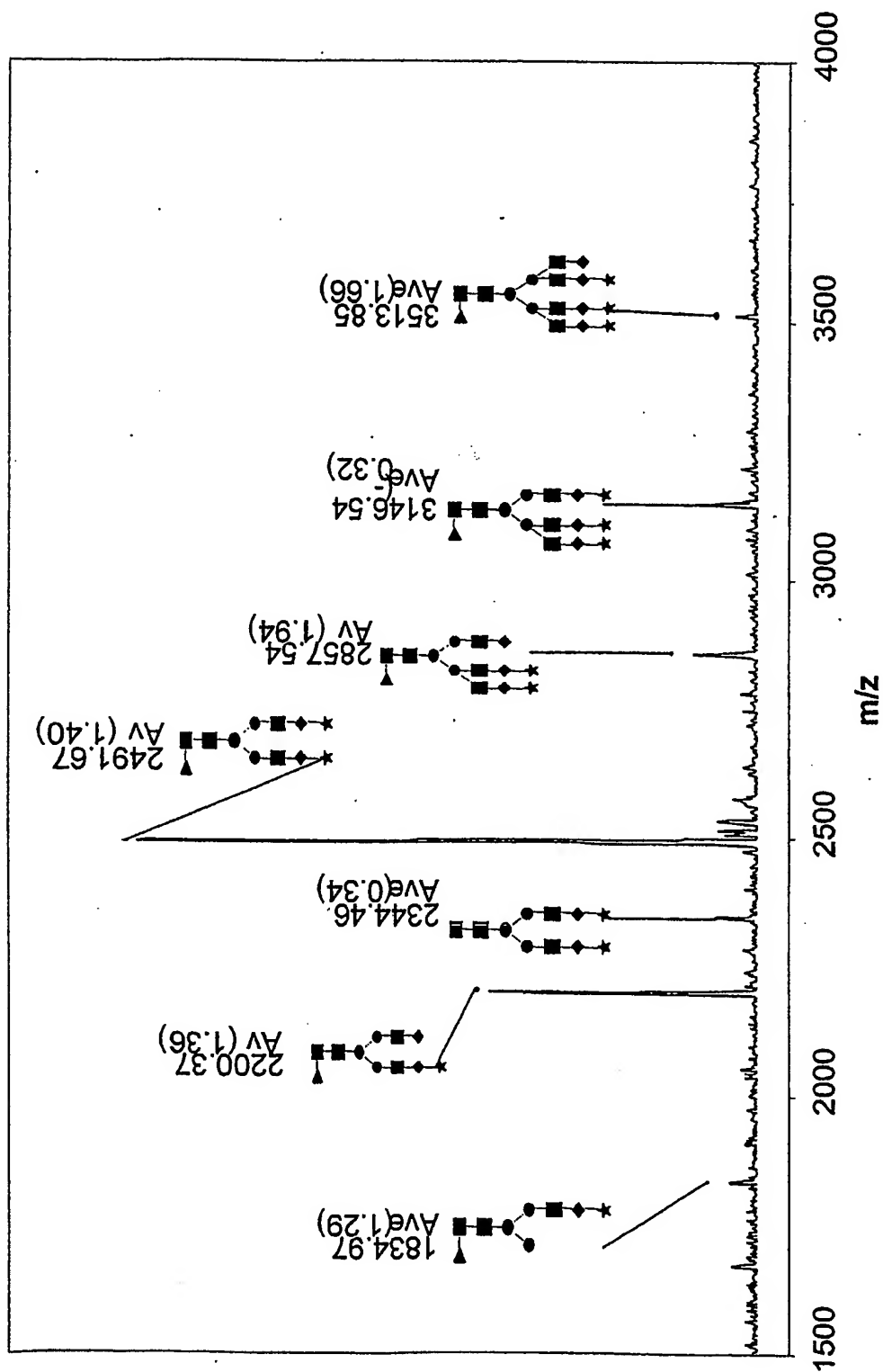


FIG. 173A

476/498

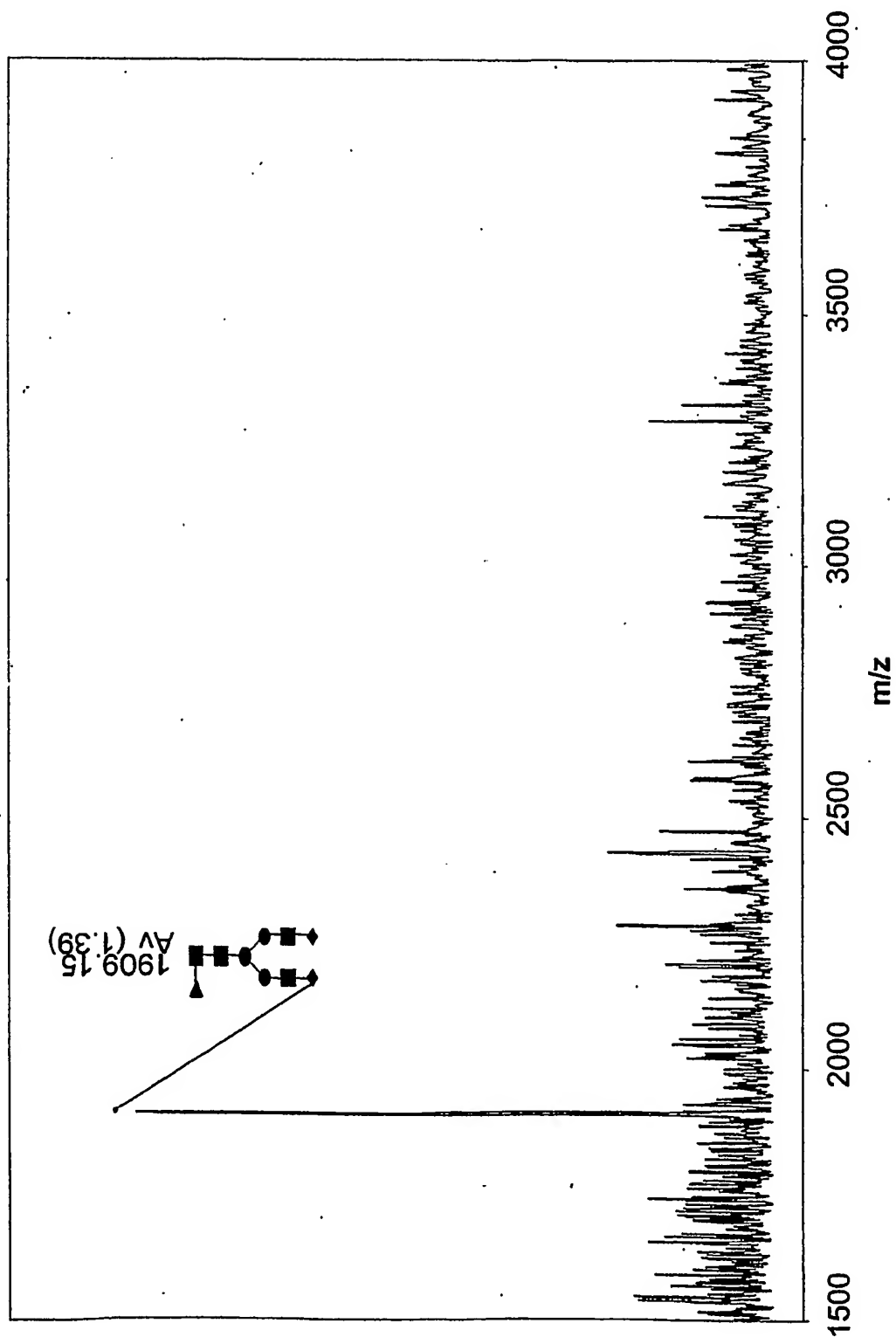


FIG. 173B

477/498

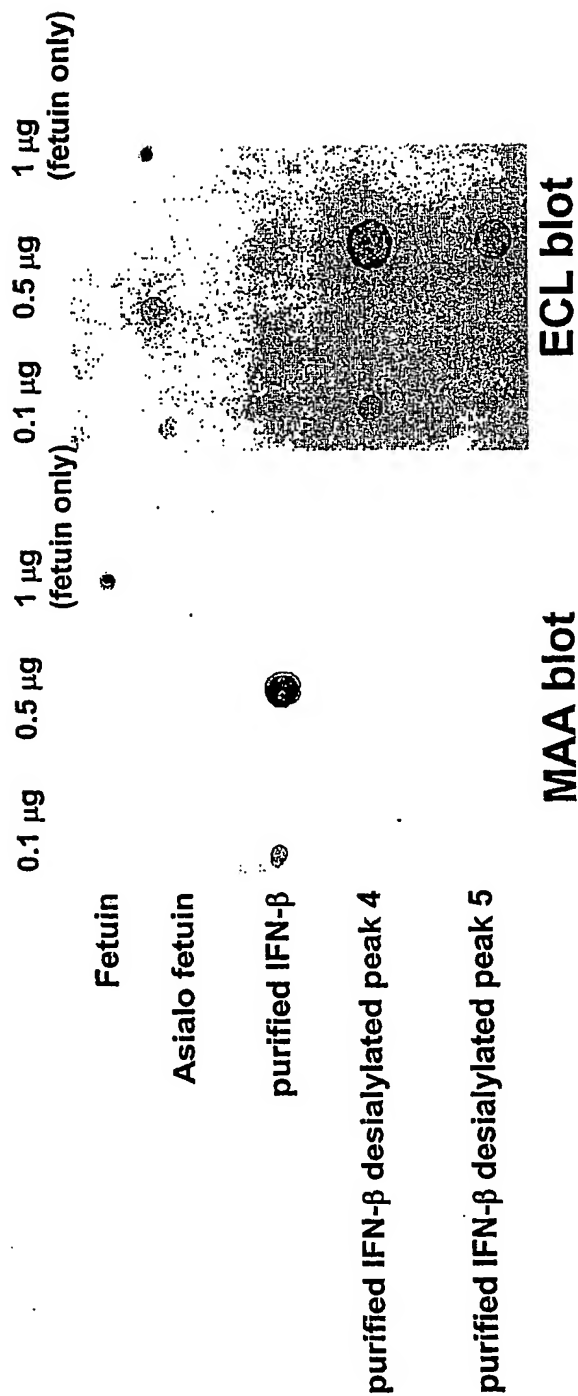


FIG. 174

478/498

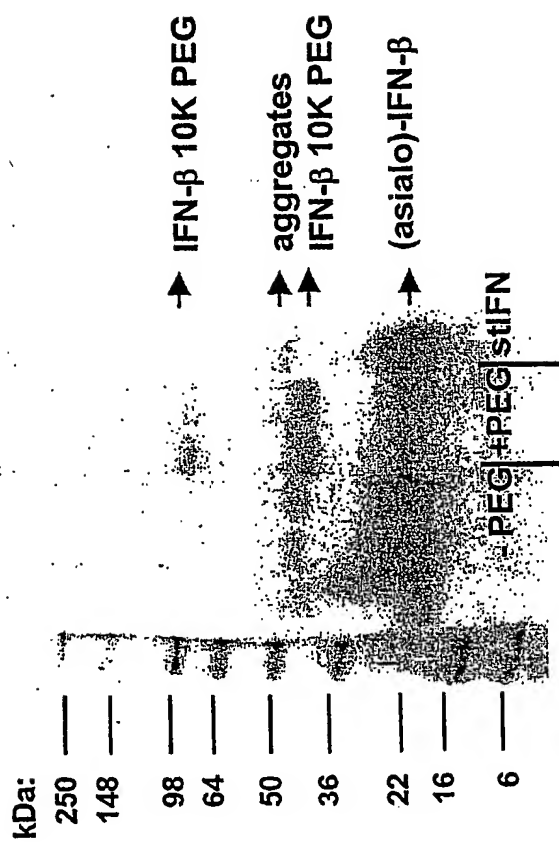
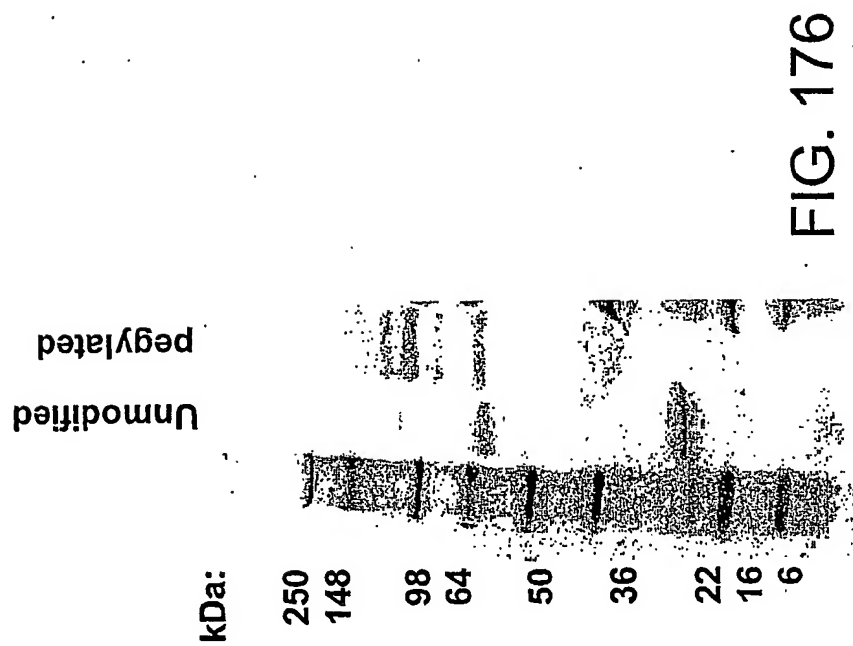


FIG. 175

479/498



480/498

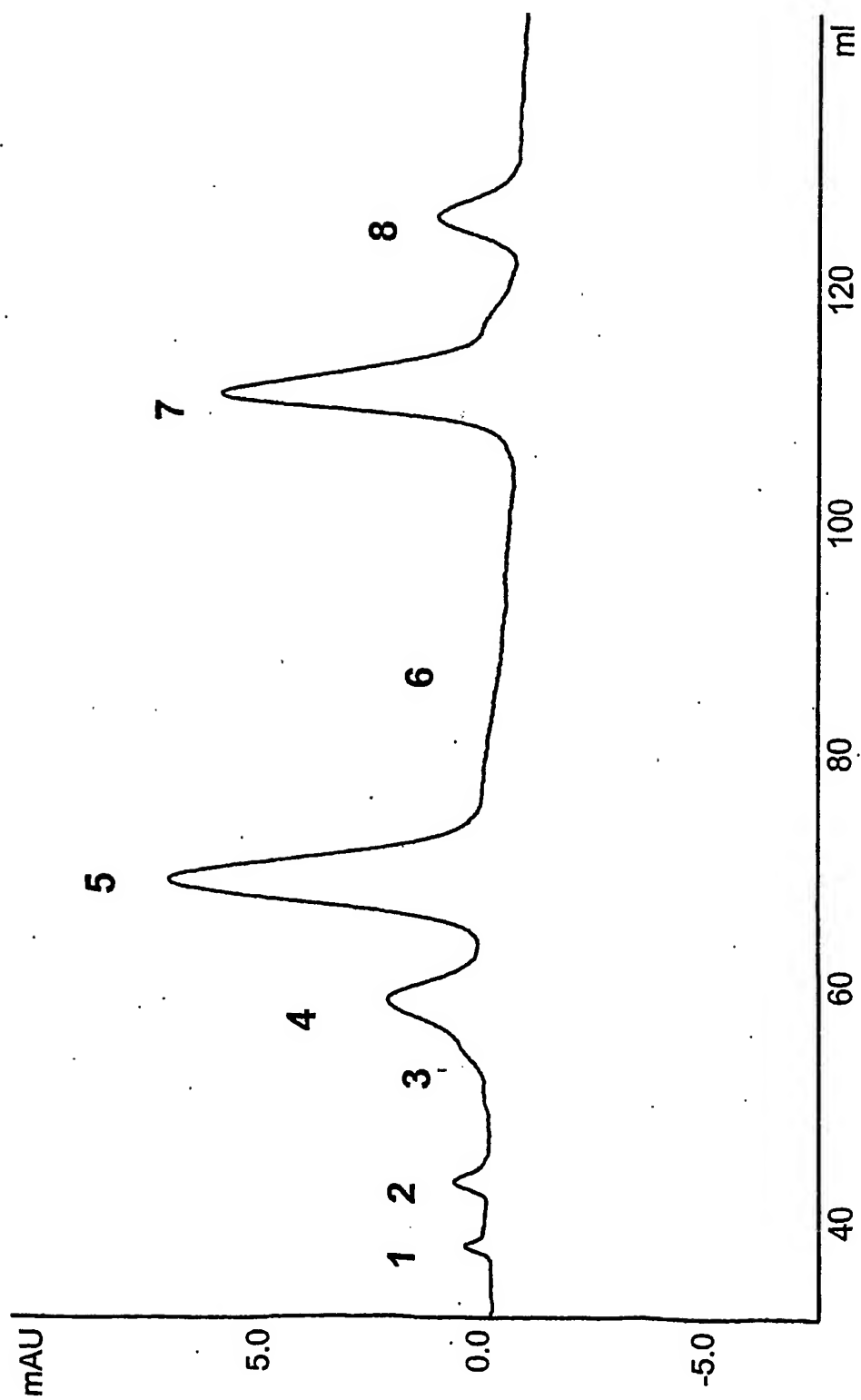


FIG. 177

481/498

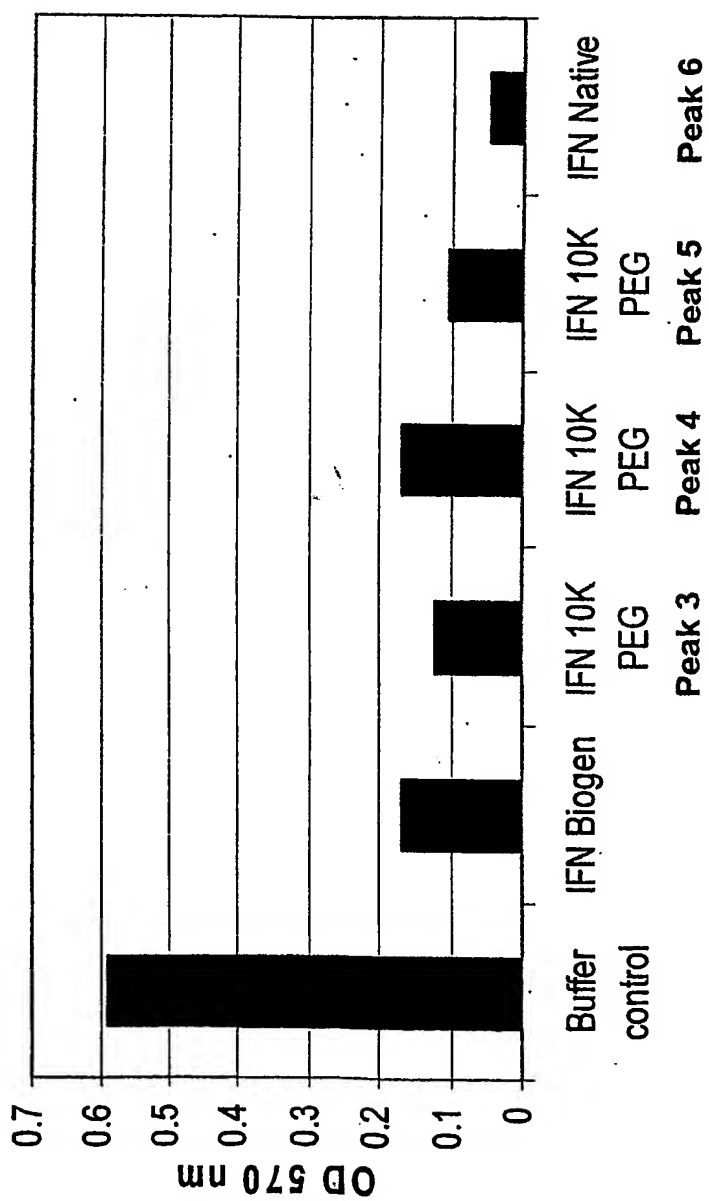


FIG. 178



482/498

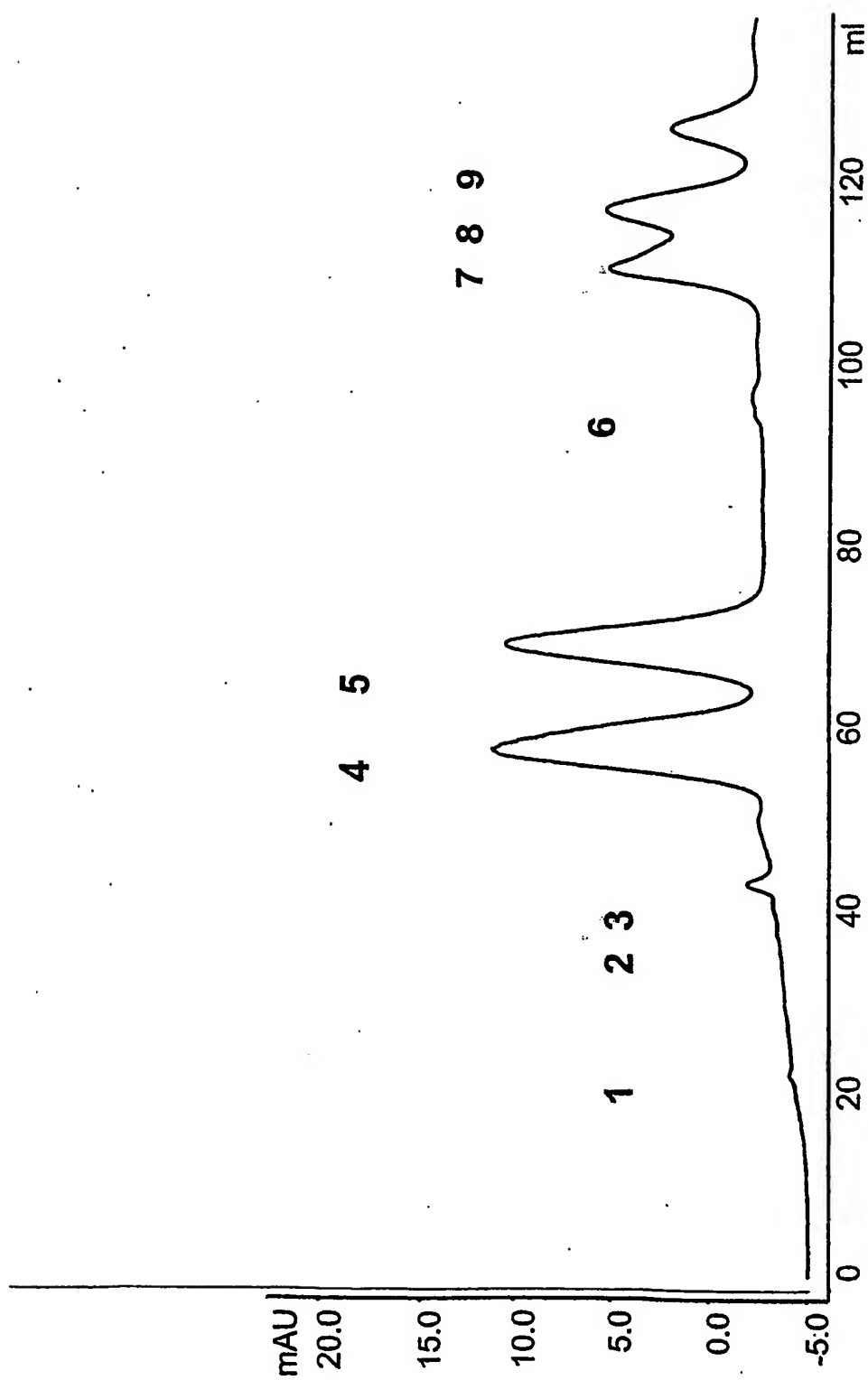


FIG. 179

483/498

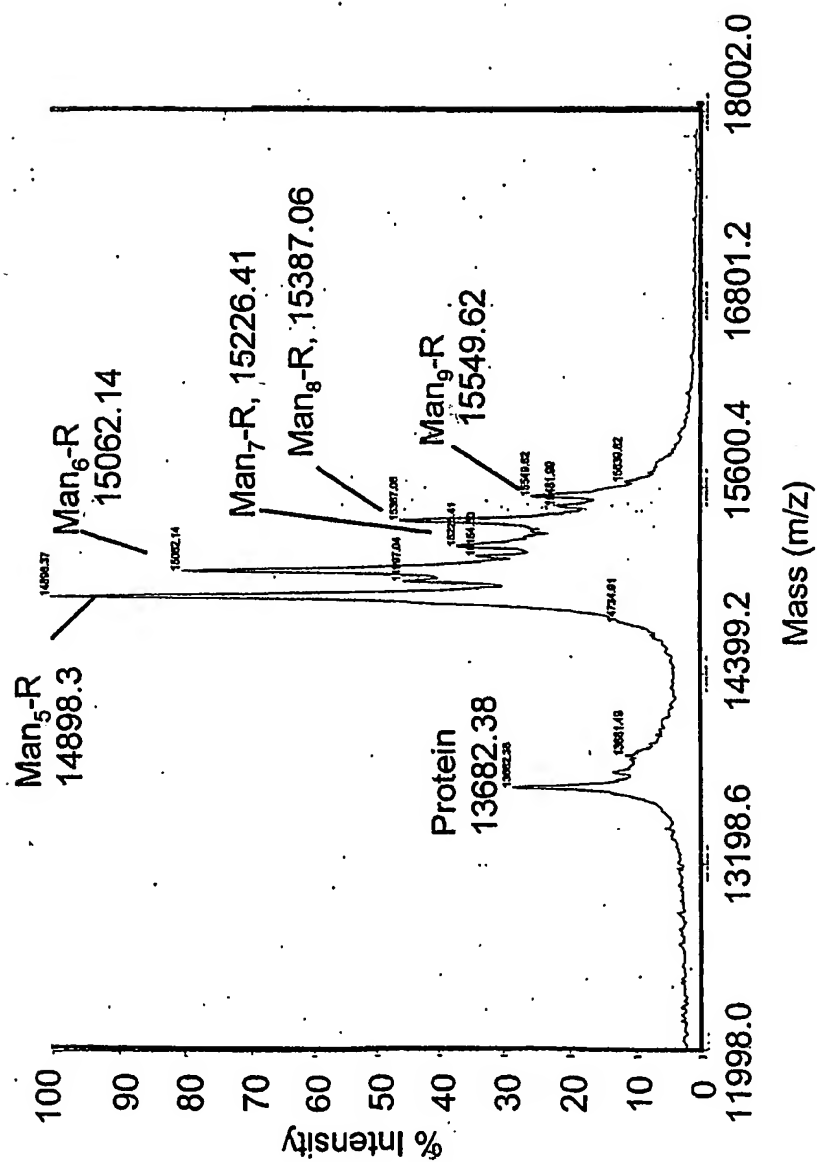


FIG. 180A

484/498

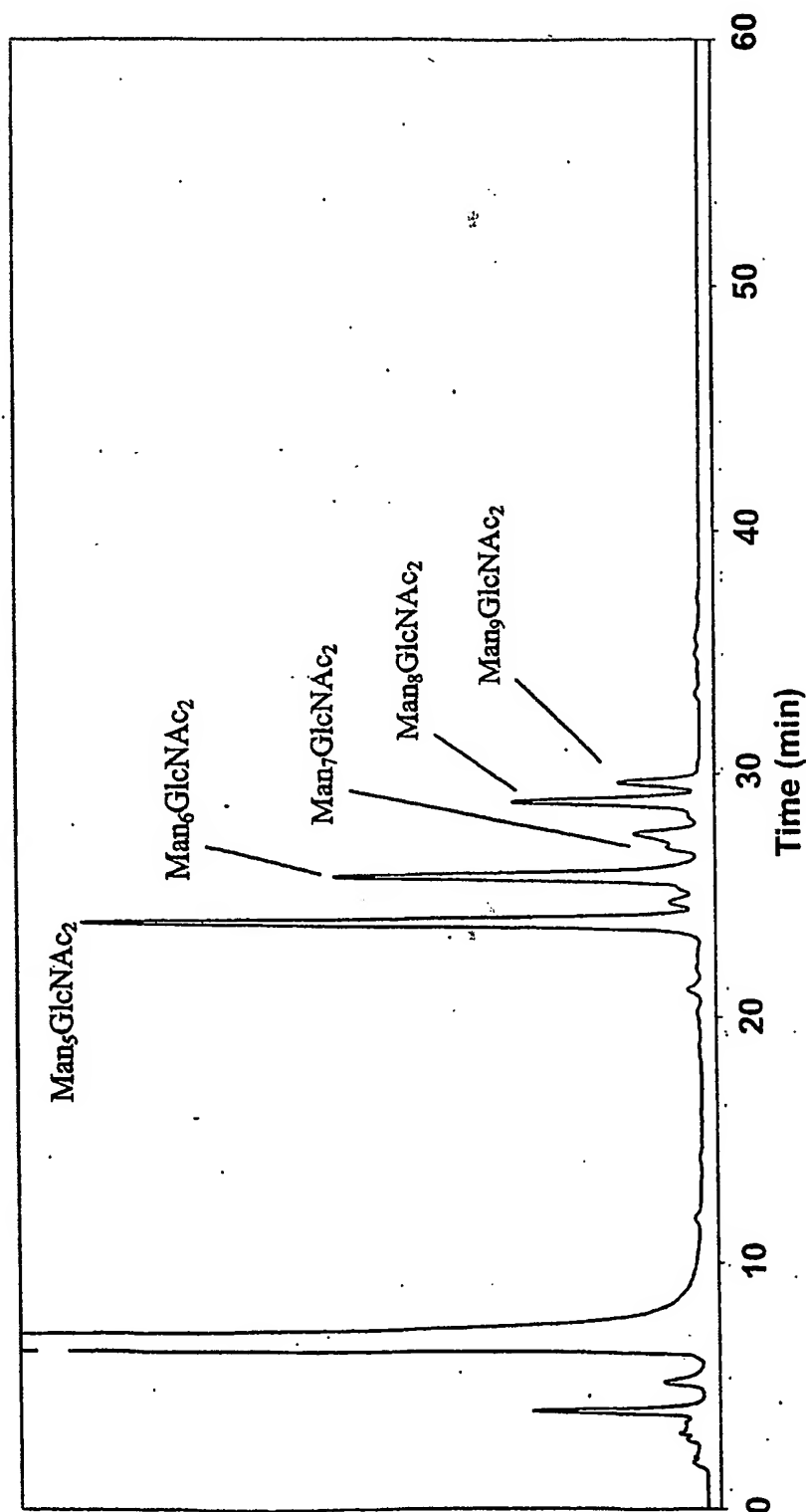


FIG. 180B

485/498

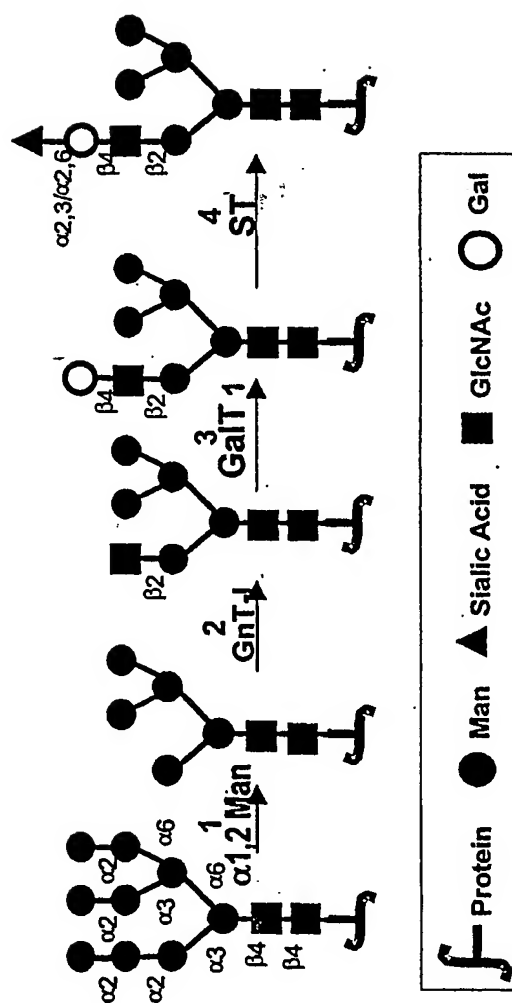


FIG. 181

486/498

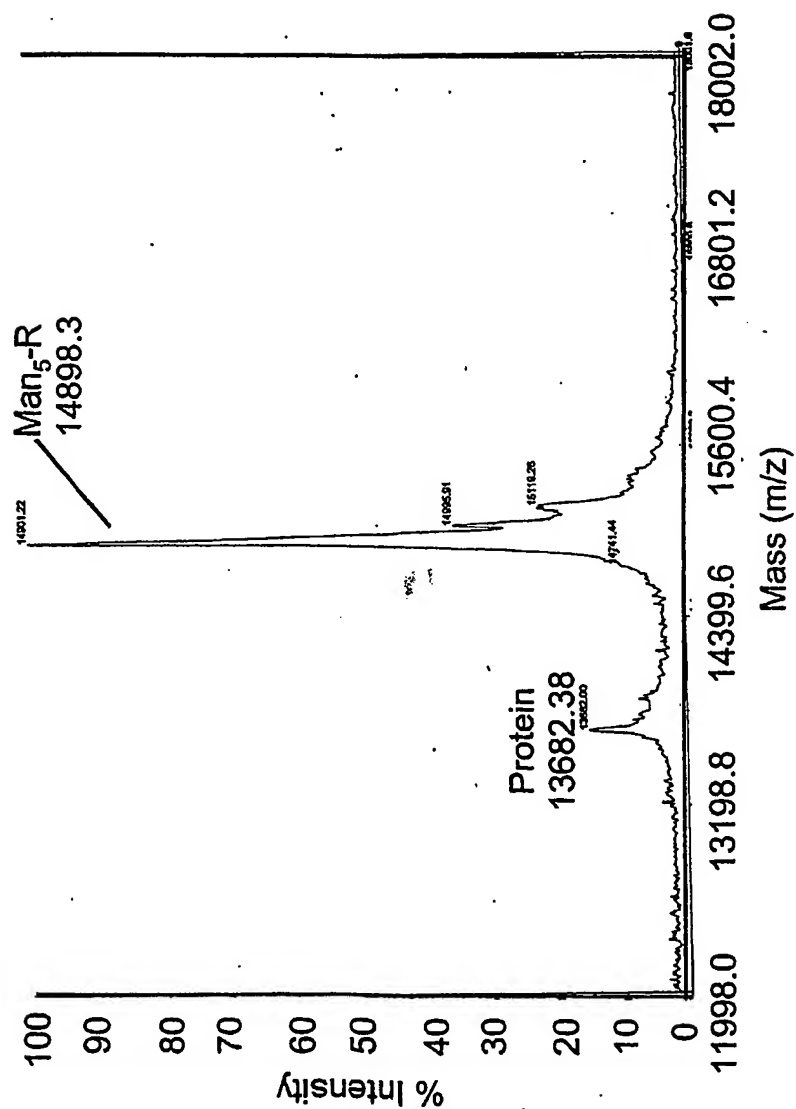


FIG. 182A

487/498

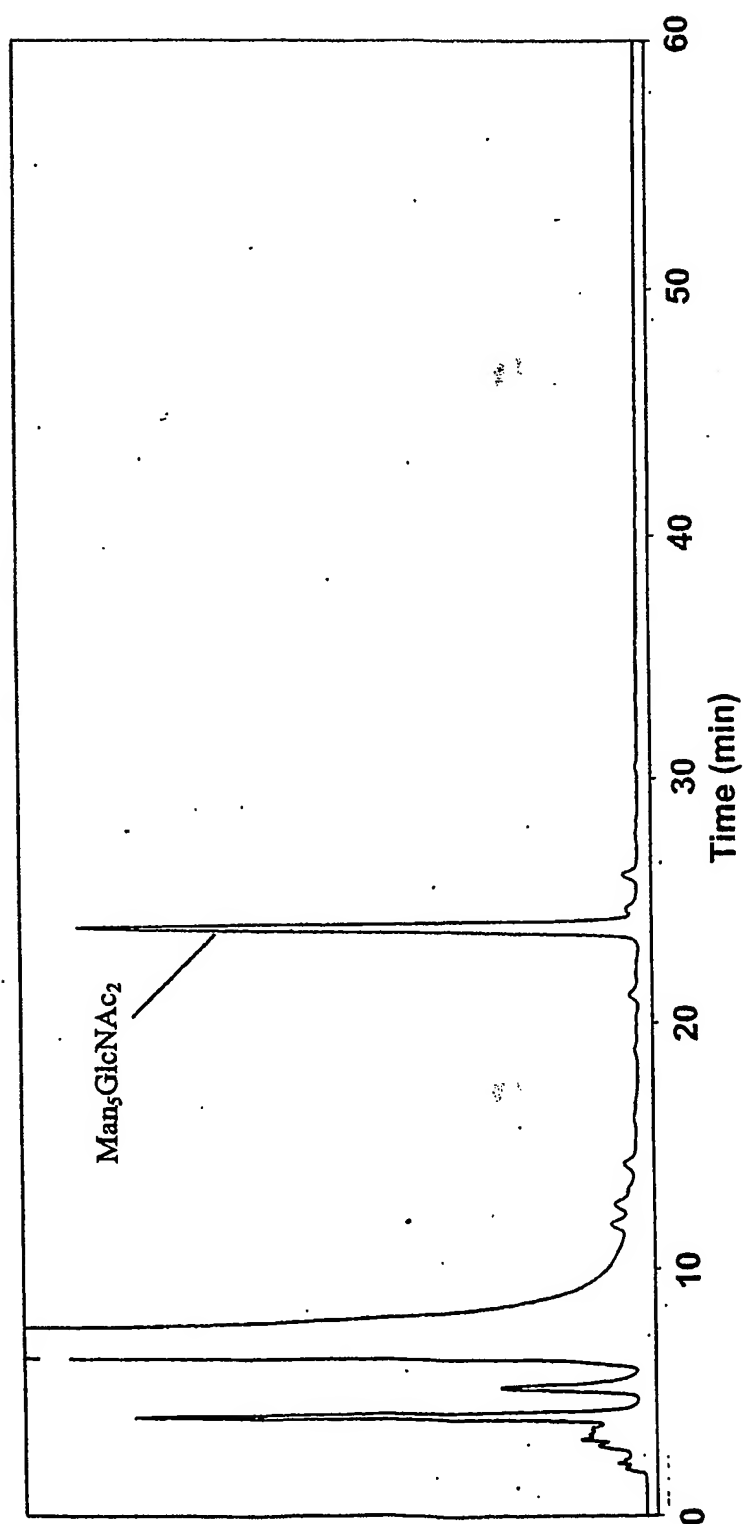


FIG. 182B

488/498

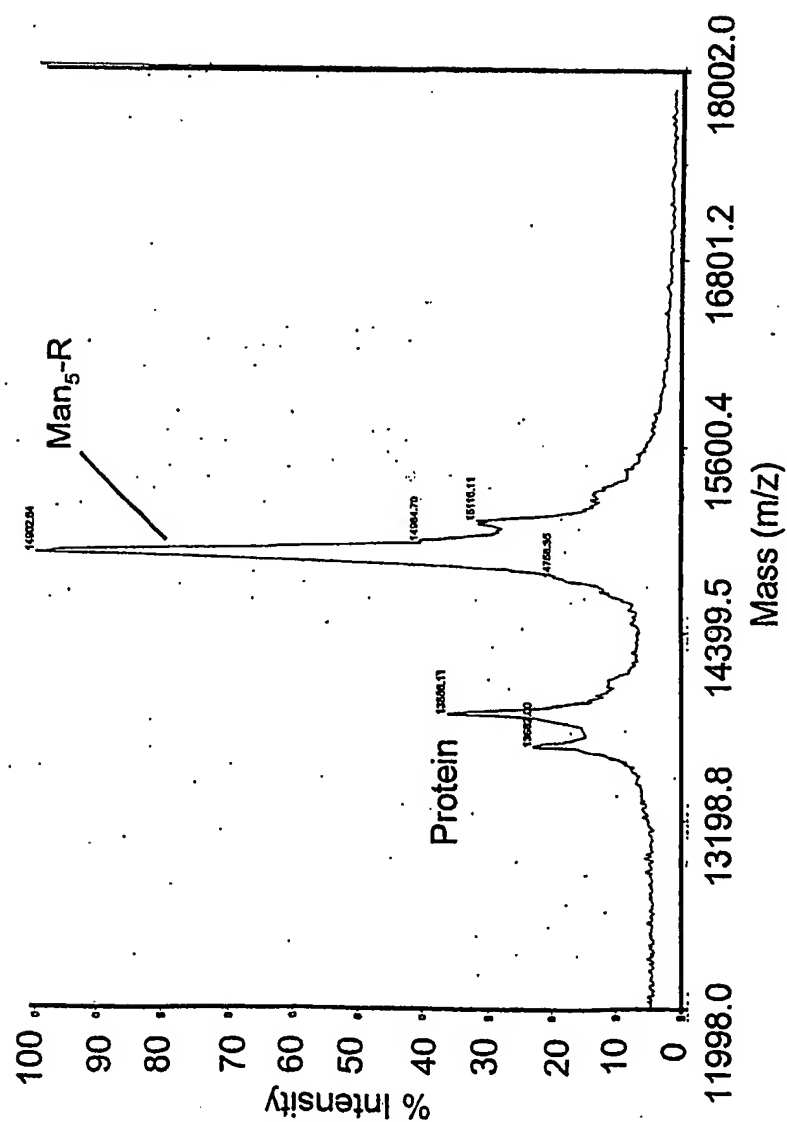


FIG. 183

489/498

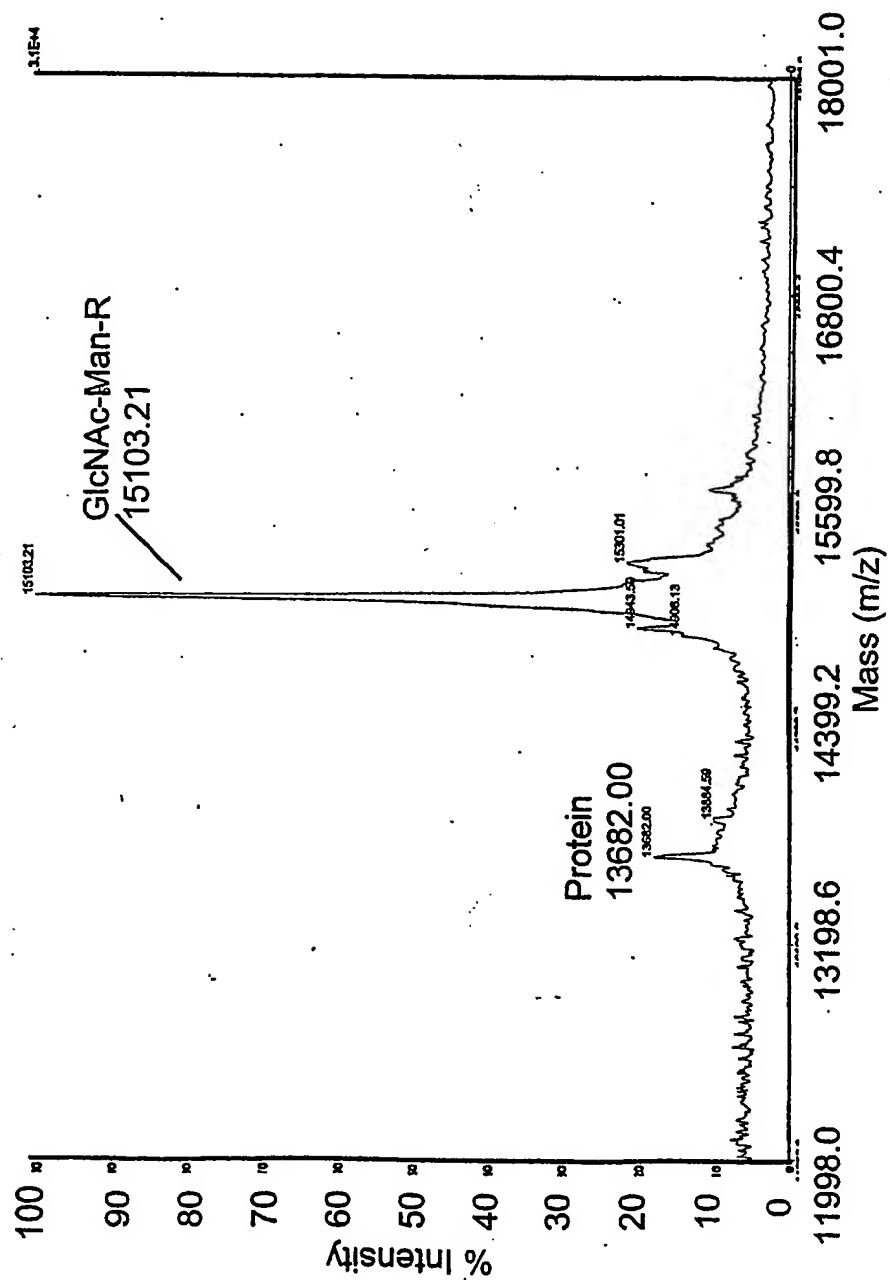


FIG. 184



490/498

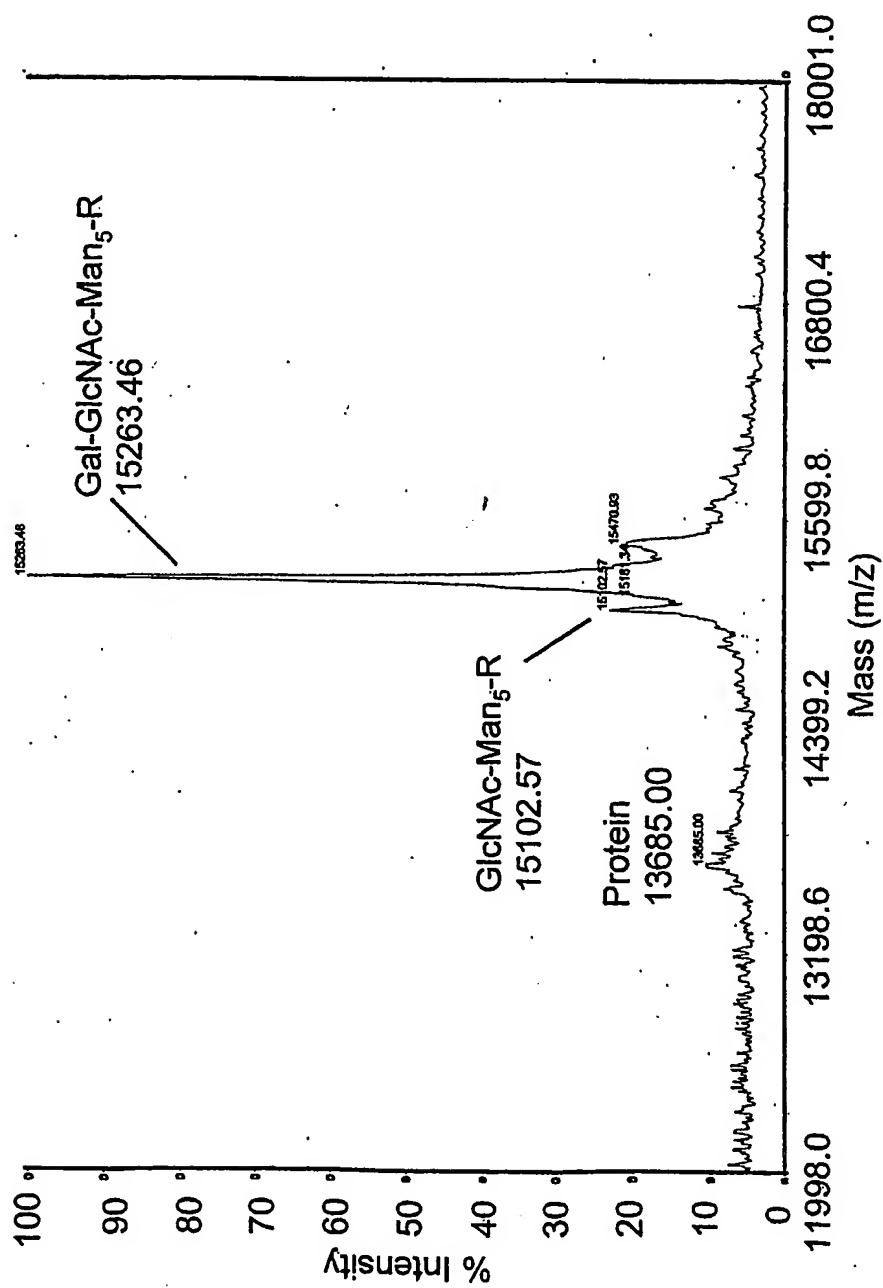


FIG. 185

491/498

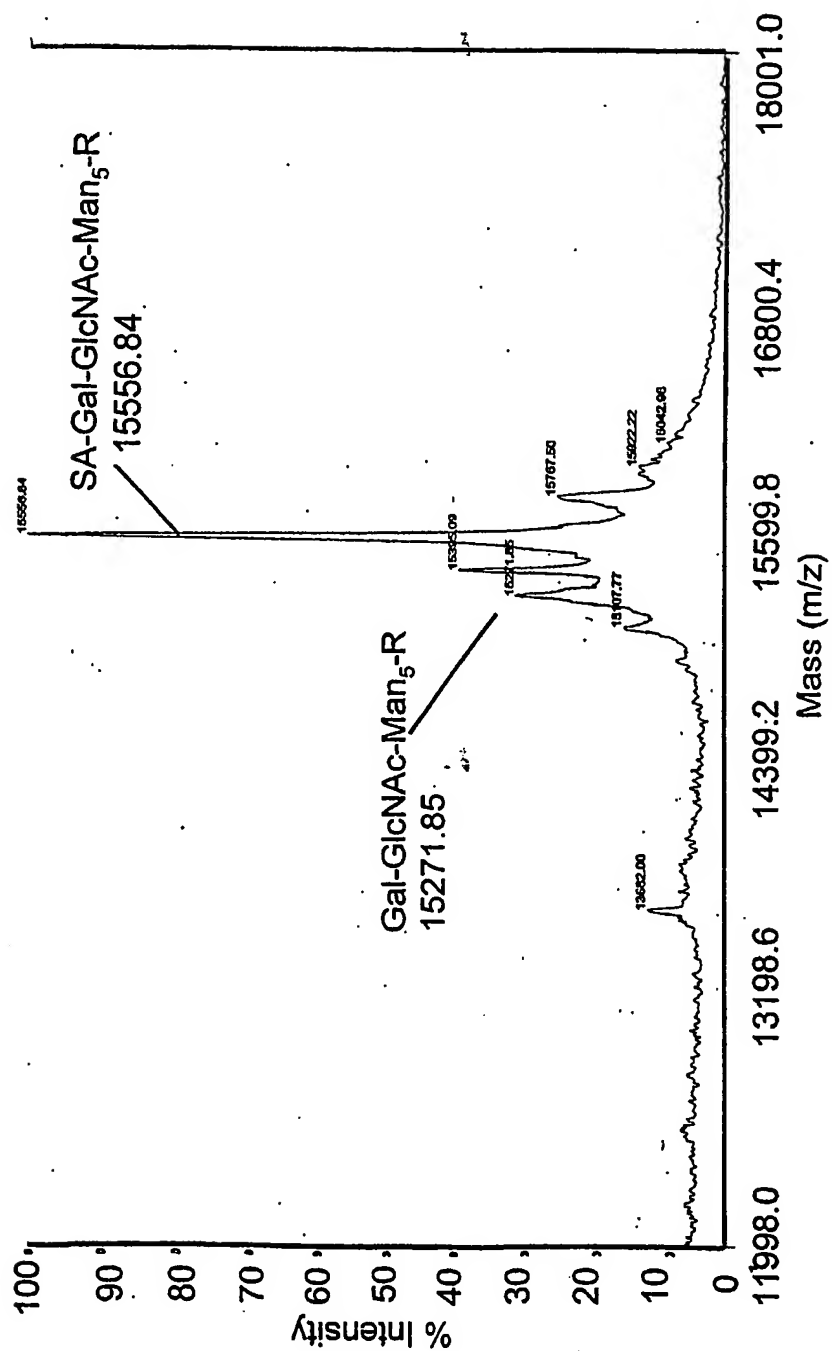


FIG. 186

492/498

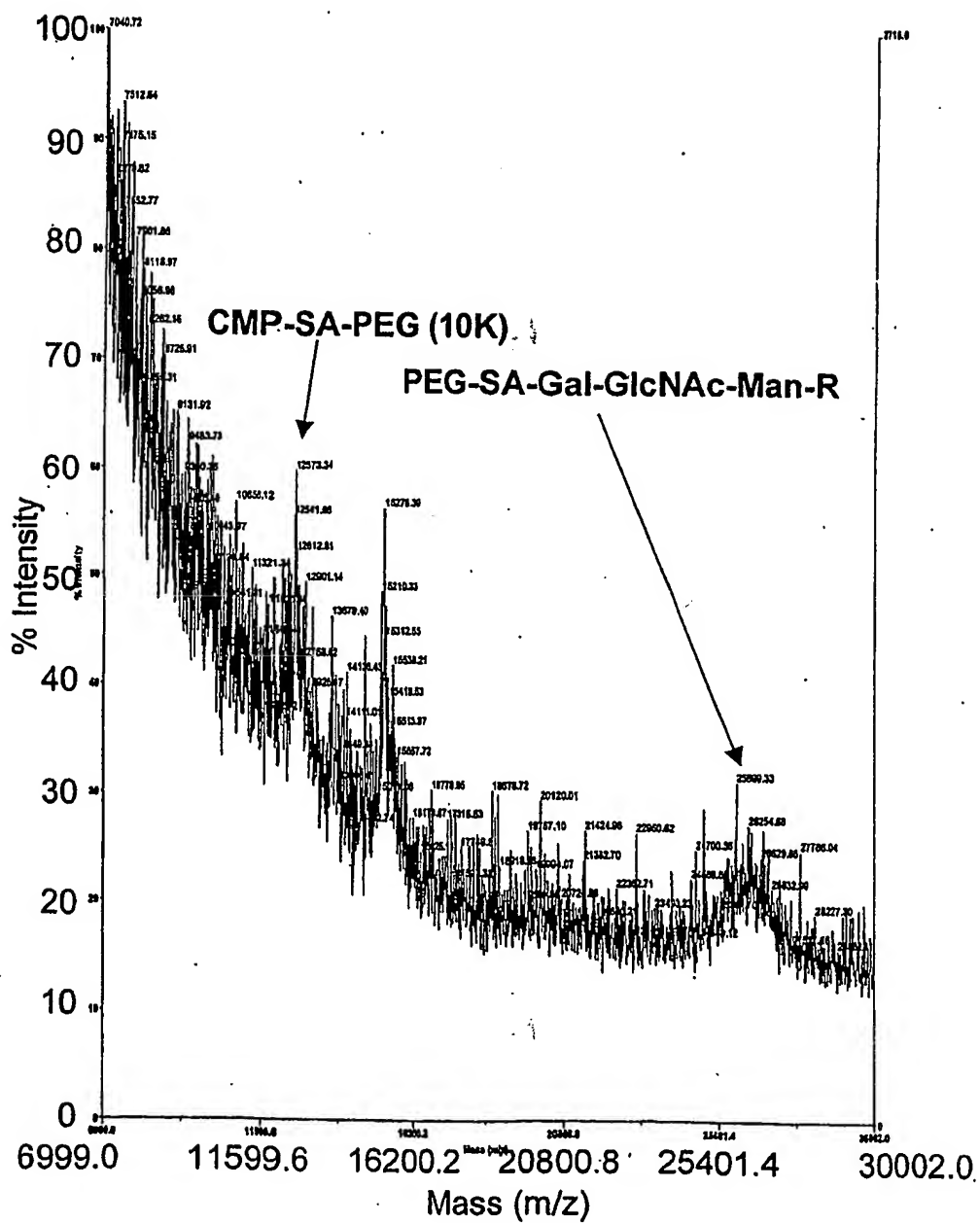
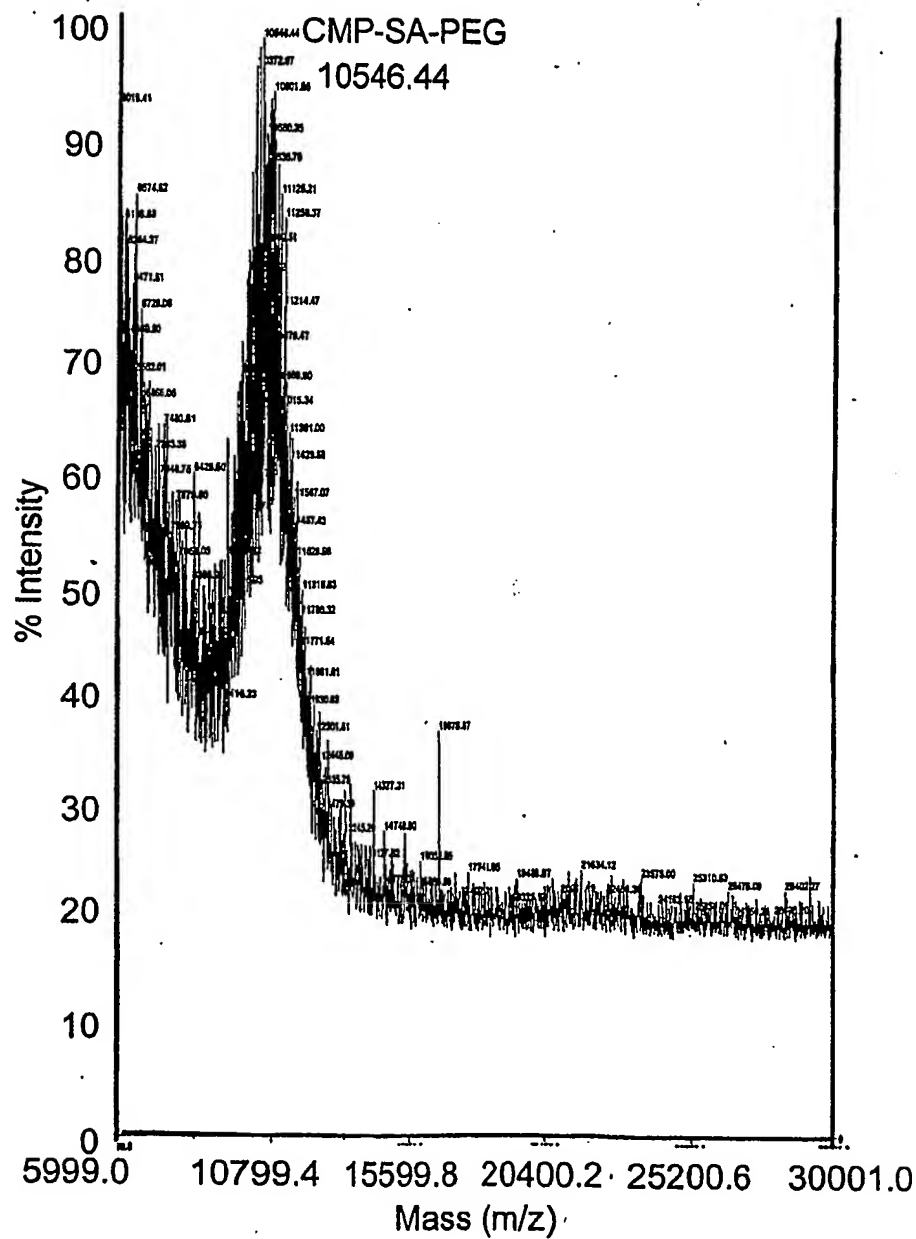


FIG. 187A

493/498



494/498

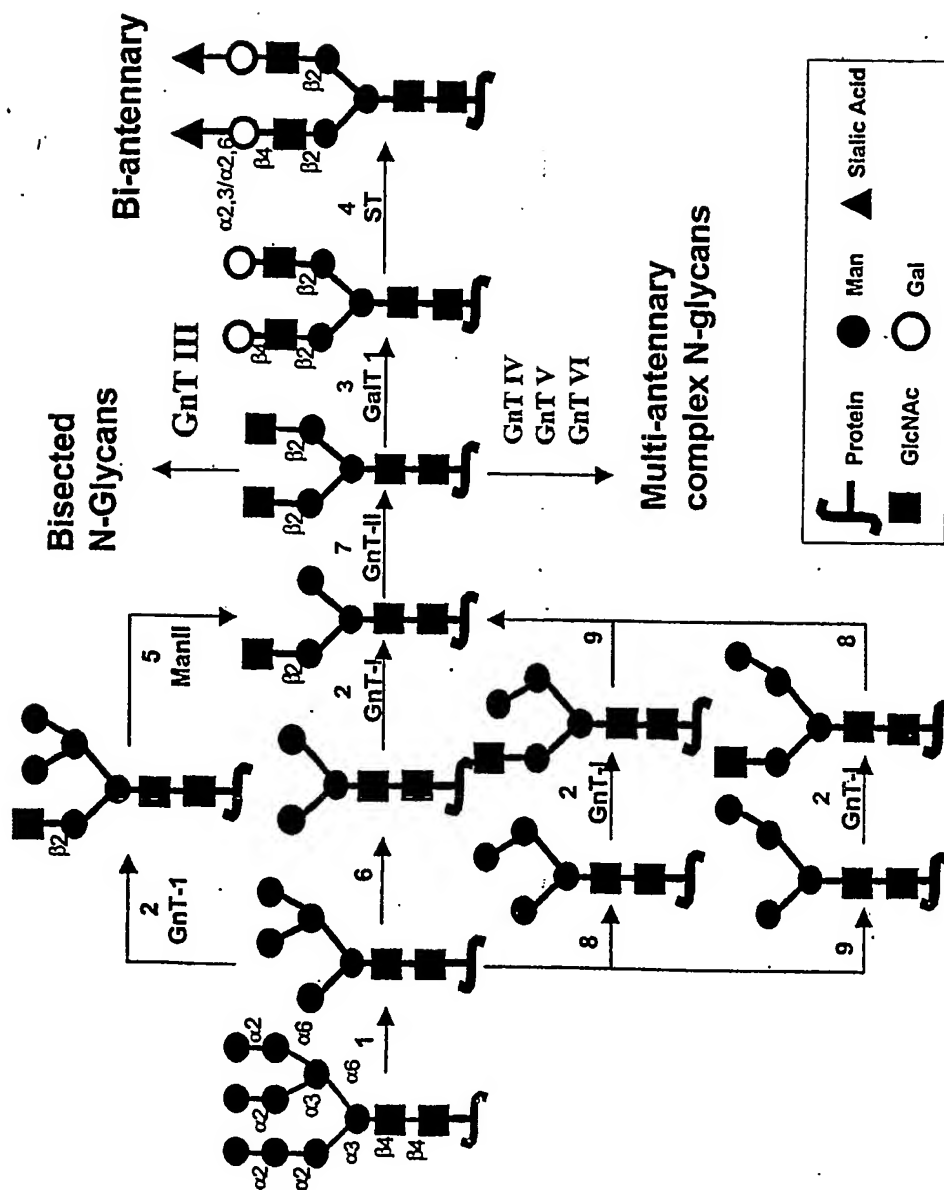


FIG. 188

495/498

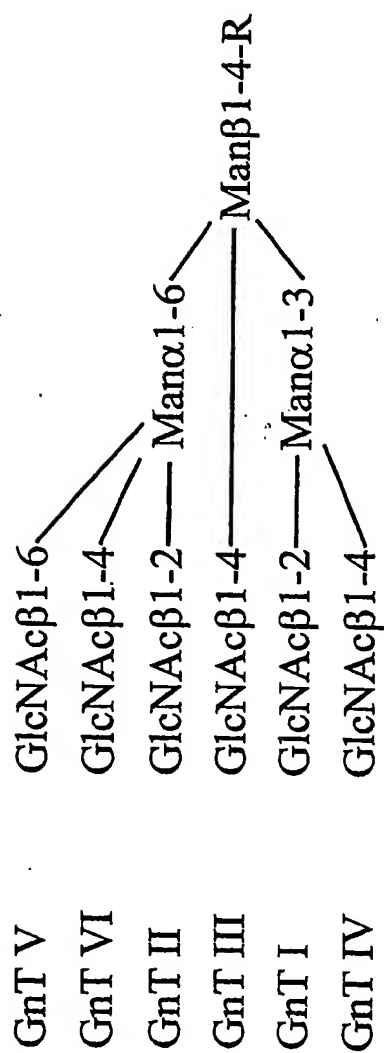


FIG. 189

496/498

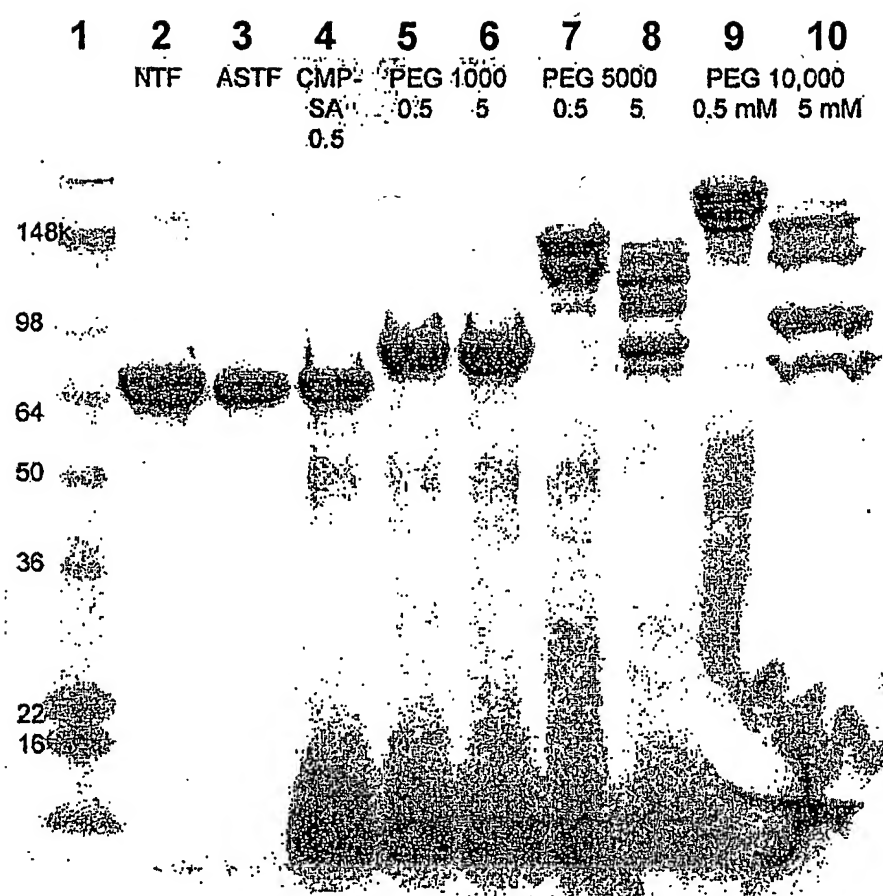


FIG. 190

497/498

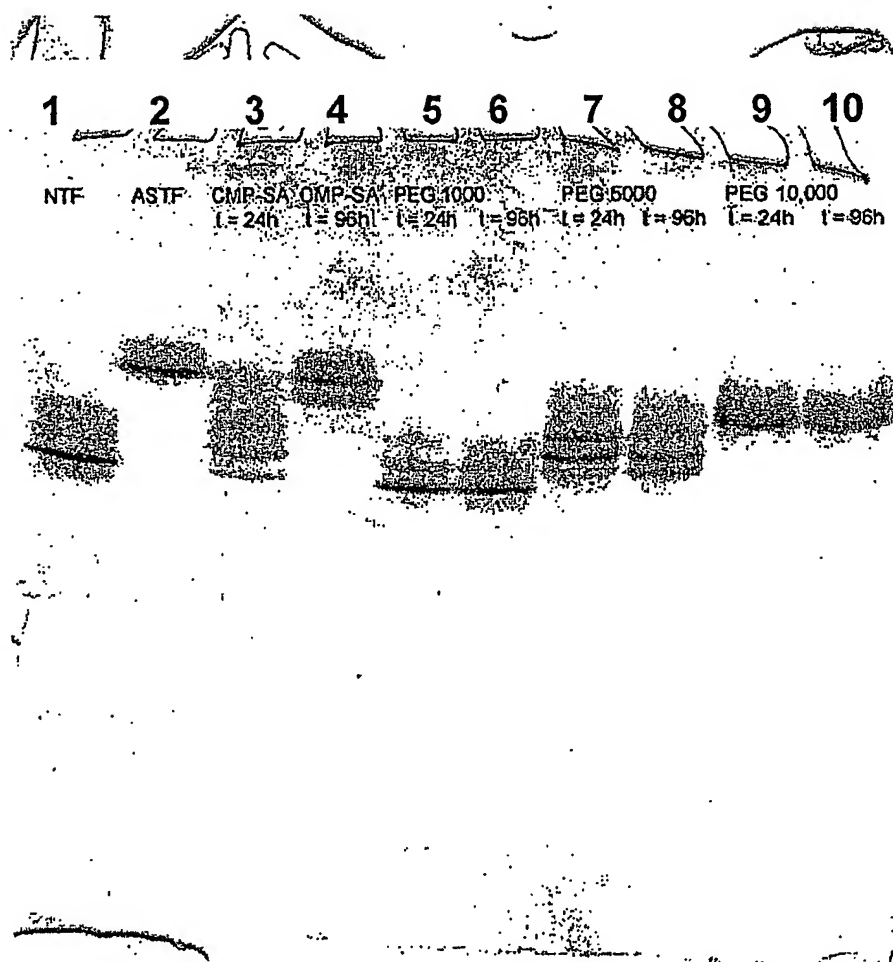
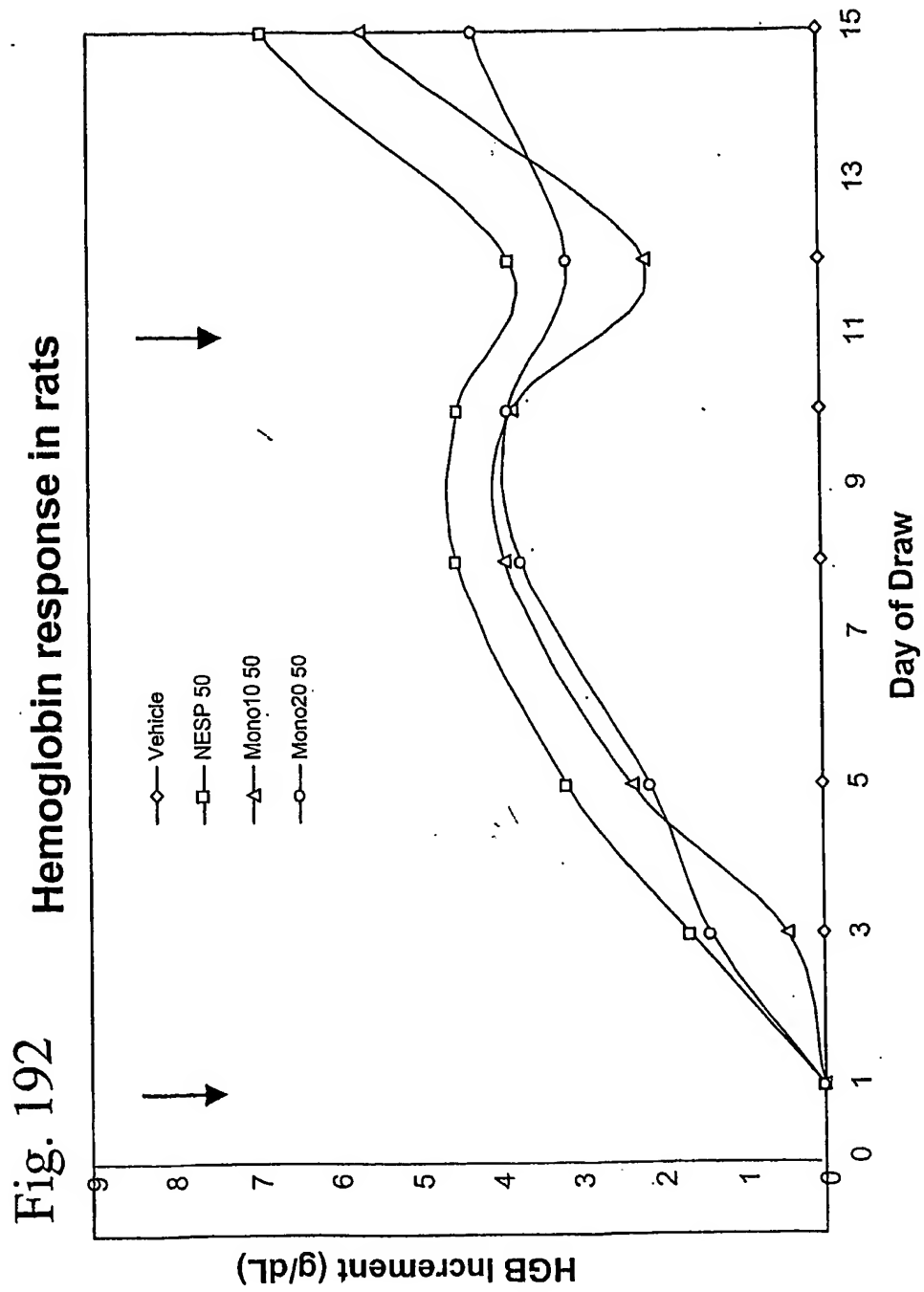


FIG. 191



498/498



## SEQUENCE LISTING

<110> Neose Technologies, Inc.  
 DeFrees, Shawn  
 Zopf, David  
 Bayer, Robert  
 Hakes, David  
 Chen, Xi  
 Bowe, Caryne

<120> ERYTHROPOIETIN: REMODELING AND GLYCOCONJUGATION OF  
 ERYTHROPOIETIN

<130> 040853-01-5083WO

<150> PCT/US02/32263  
 <151> 2002-10-09

<150> US 10/287,994  
 <151> 2002-11-5

<150> US 10/360,770  
 <151> 2003-01-06

<150> US 10/369,779  
 <151> 2003-03-17

<150> US 10/410,945  
 <151> 2003-04-09

<160> 75

<170> PatentIn version 3.2

<210> 1  
 <211> 525  
 <212> DNA  
 <213> Homo sapiens

<400> 1  
 acccccctgg gccctgccag ctccctgccc cagagcttcc tgetcaagtg cttagagcaa 60  
 gtgaggaaga tccagggcga tggcgcagcg ctccaggaga agctgtgtgc cacctacaag 120  
 ctgtgccacc ccgaggagct ggtgctgctc ggacactctc tgggcatccc ctgggctccc 180  
 ctgagcagct gccccagcca ggccctgcag ctggcagggt gcttgagcca actccatagc 240  
 ggcccttttc tctaccaggg gctcctgcag gccctggaag ggatctcccc cgagttgggt 300  
 cccaccttgg acacactgca gctggacgct gccgactttg ccaccacat ctggcagcag 360  
 atggaagaac tgggaatggc ccctgccctg cagcccaccc aggggtgcat gccggccttc 420  
 gcctctgctt tccagcgccg ggcaggagggt gtcttggttg cctcccatct gcagagcttc 480  
 ctggaggtgt cgtaccgcgt tctacgccac cttgcccagc cctga 525

<210> 2  
 <211> 174  
 <212> PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 2

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys  
 1 5 10 15  
 Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
 20 25 30  
 Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val  
 35 40 45  
 Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys  
 50 55 60  
 Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser  
 65 70 75 80  
 Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser  
 85 90 95  
 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp  
 100 105 110  
 Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro  
 115 120 125  
 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe  
 130 135 140  
 Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe  
 145 150 155 160  
 Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro  
 165 170

&lt;210&gt; 3

&lt;211&gt; 1733

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 3

gcgcctctta tgtaccaca aaaatctatt ttcaaaaaag ttgctctaag aatatagtta 60  
 tcaagttaag taaaatgtca atagcctttt aatttaattt ttaattgttt tatcattctt 120  
 tgcaataata aaacattaac tttatacttt ttaatttaat gtatagaata gagatataca 180  
 taggatatgt aaatagatac acagtgtata tgtgattaaa atataatggg agattcaatc 240  
 agaaaaaagt ttctaataaag gctctggggg aaaagaggaa ggaaacaata atgaaaaaaa 300  
 tgtggtgaga aaaacagctg aaaacccatg taaagagtgt ataaagaaag caaaaagaga 360  
 agtagaaagt aacacagggg catttggaat atgtaaacga gtatgttccc tatttaaggc 420  
 taggcacaaa gcaaggtctt cagagaacct ggagcctaag gtttaggctc acccatttca 480  
 accagtctag cagcatctgc aacatctaca atggccttga cctttgcttt actggtggcc 540  
 ctctctggtgc tcagctgcaa gtcaagctgc tctgtgggct gtgatctgcc tcaaaccac 600

agcctgggta gcaggaggac cttgatgctc ctggcacaga tgaggagaat ctctcttttc 660  
 tcctgcttga aggacagaca tgacttttga tttccccagg aggagtttgg caaccagttc 720  
 caaaaggctg aaaccatccc tgtcctccat gagatgatcc agcagatctt caatctcttc 780  
 agcacaaagg actcatctgc tgcttgggat gagaccctcc tagacaaatt ctacactgaa 840  
 ctctaccagc agctgaatga cctggaagcc tgtgtgatac aggggggtggg ggtgacagag 900  
 actccccga tgaaggagga ctccattctg gctgtgagga aatacttcca aagaatcact 960  
 ctctatctga aagagaagaa atacagccct tgtgcctggg aggttgtcag agcagaaatc 1020  
 atgagatctt tttctttgtc aacaaacttg caagaaagtt taagaagtaa ggaatgaaaa 1080  
 ctggttcaac atggaaatga ttttcattga ttctgtatgcc agctcacctt tttatgatct 1140  
 gccatttcaa agactcatgt ttctgctatg accatgacac gatttaaatac ttttcaaatg 1200  
 tttttaggag tattaatcaa cattgtattc agctcttaag gcactagtcc cttacagagg 1260  
 accatgctga ctgatccatt atctatttaa atatttttaa aatattattt atttaactat 1320  
 ttataaaaca acttattttt gttcatatta tgtcatgtgc acctttgcac agtggttaat 1380  
 gtaataaaat gtgttctttg tatttggtaa atttattttg tgttgttcat tgaacttttg 1440  
 ctatggaact tttgtacttg tttattcttt aaaatgaaat tccaagccta attgtgcaac 1500  
 ctgattacag aataactggg acacttcatt tgtccatcaa tattatattc aagatataag 1560  
 taaaaataaa ctttctgtaa accaagttgt atgttgtact caagataaca ggggtgaacct 1620  
 aacaaataca attctgctct cttgtgtatt tgatttttgt atgaaaaaaaa ctaaaaatgg 1680  
 taatcatact taattatcag ttatggtaaa tggatatgaag agaagaagga acg 1733

<210> 4  
 <211> 188  
 <212> PRT  
 <213> Homo sapiens

<400> 4  
 Met Ala Leu Thr Phe Ala Leu Leu Val Ala Leu Leu Val Leu Ser Cys  
 1 5 10 15  
 Lys Ser Ser Cys Ser Val Gly Cys Asp Leu Pro Gln Thr His Ser Leu  
 20 25 30  
 Gly Ser Arg Arg Thr Leu Met Leu Leu Ala Gln Met Arg Arg Ile Ser  
 35 40 45  
 Leu Phe Ser Cys Leu Lys Asp Arg His Asp Phe Gly Phe Pro Gln Glu  
 50 55 60  
 Glu Phe Gly Asn Gln Phe Gln Lys Ala Glu Thr Ile Pro Val Leu His  
 65 70 75 80  
 Glu Met Ile Gln Gln Ile Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser

85 90 95

Ala Ala Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr  
100 105 110

Gln Gln Leu Asn Asp Leu Glu Ala Cys Val Ile Gln Gly Val Gly Val  
115 120 125

Thr Glu Thr Pro Leu Met Lys Glu Asp Ser Ile Leu Ala Val Arg Lys  
130 135 140

Tyr Phe Gln Arg Ile Thr Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro  
145 150 155 160

Cys Ala Trp Glu Val Val Arg Ala Glu Ile Met Arg Ser Phe Ser Leu  
165 170 175

Ser Thr Asn Leu Gln Glu Ser Leu Arg Ser Lys Glu  
180 185

<210> 5  
<211> 757  
<212> DNA  
<213> Homo sapiens

<400> 5  
atgaccaaca agtgtctcct ccaaattgct ctctgttgt gcttctccac tacagctctt 60  
tccatgagct acaacttgct tggattccta caaagaagca gcaattttca gtgtcagaag 120  
ctctgtggc aattgaatgg gaggcttgaa tattgcctca aggacaggat gaactttgac 180  
atccctgagg agattaagca gctgcagcag ttccagaagg aggacgccgc attgaccatc 240  
tatgagatgc tccagaacat ctttgctatt ttcagacaag attcatctag cactggctgg 300  
aatgagacta ttgttgagaa cctctggct aatgtctatc atcagataaa ccatctgaag 360  
acagtcctgg aagaaaaact ggagaaagaa gattttacca ggggaaaact catgagcagt 420  
ctgcacctga aaagatatta tgggaggatt ctgcattacc tgaaggccaa ggagtacagt 480  
cactgtgcct ggaccatagt cagagtggaa atcctaagga acttttactt cattaacaga 540  
cttacagggtt acctccgaaa ctgaagatct cctagcctgt cctctggga ctggacaatt 600  
gcttcaagca ttcttcaacc agcagatgct gttaagtga ctgatggcta atgtactgca 660  
aatgaaagga cactagaaga ttttgaaatt ttattataat tatgagttat ttttatttat 720  
ttaaatttta ttttgaaaa taaattattt ttggtgc 757

<210> 6  
<211> 187  
<212> PRT  
<213> Homo sapiens

<400> 6  
Met Thr Asn Lys Cys Leu Leu Gln Ile Ala Leu Leu Leu Cys Phe Ser  
1 5 10 15

Thr Thr Ala Leu Ser Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg  
 20 25 30  
 Ser Ser Asn Phe Gln Cys Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg  
 35 40 45  
 Leu Glu Tyr Cys Leu Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu  
 50 55 60  
 Ile Lys Gln Leu Gln Gln Phe Gln Lys Glu Asp Ala Ala Leu Thr Ile  
 65 70 75 80  
 Tyr Glu Met Leu Gln Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser  
 85 90 95  
 Ser Thr Gly Trp Asn Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val  
 100 105 110  
 Tyr His Gln Ile Asn His Leu Lys Thr Val Leu Glu Glu Lys Leu Glu  
 115 120 125  
 Lys Glu Asp Phe Thr Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys  
 130 135 140  
 Arg Tyr Tyr Gly Arg Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser  
 145 150 155 160  
 His Cys Ala Trp Thr Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr  
 165 170 175  
 Phe Ile Asn Arg Leu Thr Gly Tyr Leu Arg Asn  
 180 185

<210> 7  
 <211> 1332  
 <212> DNA  
 <213> Homo sapiens

<400> 7  
 atggtctccc aggccctcag gctcctctgc cttctgcttg ggcttcaggg ctgcctggct 60  
 gcagtcttcg taaccaggga ggaagccac ggcgtcctgc accggcgccg gcgcgccaac 120  
 gcgttccttg aggagctgcg gccgggctcc ctggagaggg agtgcaagga ggagcagtgc 180  
 tccttcgagg aggcccgga gatcttcaag gacgcggaga ggacgaagct gttctggatt 240  
 tcttacagtg atggggacca gtgtgcctca agtccatgcc agaatggggg ctcttgcaag 300  
 gaccagctcc agtctatat ctgcttctgc ctccctgcct tcgagggccg gaactgtgag 360  
 acgcacaagg atgaccagct gatctgtgtg aacgagaacg gcggctgtga gcagtaactgc 420  
 agtgaccaca cgggcaccaa gcgtcctgt cgggtccacg aggggtactc tctgctggca 480  
 gacgggggtg cctgcacacc cacagttgaa tatccatgtg gaaaaatacc tattctagaa 540  
 aaaagaaatg ccagcaaacc ccaaggccga attgtggggg gcaagggtgtg ccccaaaggg 600  
 gagtgtocat ggcaggtcct gttgttggtg aatggagctc agttgtgtgg ggggaccctg 660

```

atcaacacca tctgggtggt ctccgcggcc cactgtttcg acaaaatcaa gaactggagg 720
aacctgatcg cgggtgctggg cgagcacgac ctgagcgagc acgacgggga tgagcagagc 780
cggcgggtgg cgcaggtcat catccccagc acgtacgtcc cgggcaccac caaccacgac 840
atcgcgctgc tccgcctgca ccagcccgtg gtcctactg accatgtggt gccctctctgc 900
ctgcccgaac ggacgttctc tgagaggacg ctggccttcg tgcgttctc attggtcagc 960
ggctggggcc agctgctgga ccgtggcgcc acggccctgg agctcatggt gctcaacgtg 1020
ccccggctga tgaccagga ctgctgcag cagtcacgga aggtgggaga ctccccaaat 1080
atcacggagt acatgttctg tgccggctac tcggatggca gcaaggactc ctgcaagggg 1140
gacagtggag gccacatgc caccactac cggggcacgt ggtacctgac gggcatcgtc 1200
agctggggcc agggctgcgc aaccgtgggc cactttgggg tgtacaccag ggtctcccag 1260
tacatcgagt ggctgcaaaa gctcatgcgc tcagagccac gccaggagt cctcctgcga 1320
gccccatttc cc 1332

```

<210> 8  
 <211> 444  
 <212> PRT  
 <213> Homo sapiens

<400> 8  
 Met Val Ser Gln Ala Leu Arg Leu Leu Cys Leu Leu Leu Gly Leu Gln  
 1 5 10 15  
 Gly Cys Leu Ala Ala Val Phe Val Thr Gln Glu Glu Ala His Gly Val  
 20 25 30  
 Leu His Arg Arg Arg Arg Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro  
 35 40 45  
 Gly Ser Leu Glu Arg Glu Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu  
 50 55 60  
 Ala Arg Glu Ile Phe Lys Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile  
 65 70 75 80  
 Ser Tyr Ser Asp Gly Asp Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly  
 85 90 95  
 Gly Ser Cys Lys Asp Gln Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro  
 100 105 110  
 Ala Phe Glu Gly Arg Asn Cys Glu Thr His Lys Asp Asp Gln Leu Ile  
 115 120 125  
 Cys Val Asn Glu Asn Gly Gly Cys Glu Gln Tyr Cys Ser Asp His Thr  
 130 135 140  
 Gly Thr Lys Arg Ser Cys Arg Cys His Glu Gly Tyr Ser Leu Leu Ala  
 145 150 155 160  
 Asp Gly Val Ser Cys Thr Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile  
 165 170 175

Pro Ile Leu Glu Lys Arg Asn Ala Ser Lys Pro Gln Gly Arg Ile Val  
 180 185 190  
 Gly Gly Lys Val Cys Pro Lys Gly Glu Cys Pro Trp Gln Val Leu Leu  
 195 200 205  
 Leu Val Asn Gly Ala Gln Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile  
 210 215 220  
 Trp Val Val Ser Ala Ala His Cys Phe Asp Lys Ile Lys Asn Trp Arg  
 225 230 235 240  
 Asn Leu Ile Ala Val Leu Gly Glu His Asp Leu Ser Glu His Asp Gly  
 245 250 255  
 Asp Glu Gln Ser Arg Arg Val Ala Gln Val Ile Ile Pro Ser Thr Tyr  
 260 265 270  
 Val Pro Gly Thr Thr Asn His Asp Ile Ala Leu Leu Arg Leu His Gln  
 275 280 285  
 Pro Val Val Leu Thr Asp His Val Val Pro Leu Cys Leu Pro Glu Arg  
 290 295 300  
 Thr Phe Ser Glu Arg Thr Leu Ala Phe Val Arg Phe Ser Leu Val Ser  
 305 310 315 320  
 Gly Trp Gly Gln Leu Leu Asp Arg Gly Ala Thr Ala Leu Glu Leu Met  
 325 330 335  
 Val Leu Asn Val Pro Arg Leu Met Thr Gln Asp Cys Leu Gln Gln Ser  
 340 345 350  
 Arg Lys Val Gly Asp Ser Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala  
 355 360 365  
 Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly  
 370 375 380  
 Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val  
 385 390 395 400  
 Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His Phe Gly Val Tyr Thr  
 405 410 415  
 Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys Leu Met Arg Ser Glu  
 420 425 430  
 Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe Pro  
 435 440

&lt;210&gt; 9

&lt;211&gt; 1437

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 9

atgcagcgcg tgaacatgat catggcagaa tcaccaagcc tcataccat ctgcctttta 60

ggatatctac tcagtgtga atgtacagtt tttcttgatc atgaaaacgc caacaaaatt 120



ctgaatcggc caaagaggta taattcaggt aaattggaag agtttgttca agggaacctt 180  
 gagagagaat gtatggaaga aaagtgtagt tttgaagaac cacgagaagt ttttgaaaac 240  
 actgaaaaga caactgaatt ttggaagcag tatgttgatg gagatcagtg tgagtccaat 300  
 ccatgtttta atggcggcag ttgcaaggat gacattaatt cctatgaatg ttgggtgtccc 360  
 tttggatttg aaggaaagaa ctgtgaatta gatgtaacat gtaacattaa gaatggcaga 420  
 tgcgagcagt tttgtaaaaa tagtgctgat aacaagggtg tttgctcctg tactgagggg 480  
 tatcgacttg cagaaaaacca gaagtcctgt gaaccagcag tgccatttcc atgtggaaga 540  
 gtttctgttt cacaaacttc taagctcacc cgtgctgagg ctgtttttcc tgatgtggac 600  
 tatgtaaatc ctactgaagc tgaaaccatt ttggataaca tcaactcaagg cacccaatca 660  
 tttaatgact tcaactcgggt tgttggtgga gaagatgcca aaccagggtca attcccttgg 720  
 caggttgttt tgaatggtaa agttgatgca ttctgtggag gctctatcgt taatgaaaaa 780  
 tggattgtaa ctgctgcccc ctgtgttgaa actggtgtta aaattacagt tgcgcaggt 840  
 gaacataata ttgaggagac agaacataca gagcaaaagc gaaatgtgat tcgagcaatt 900  
 attcctcacc acaactacaa tgcagctatt aataagtaca accatgacat tgccttctg 960  
 gaactggacg aacccttagt gctaaacagc tacgttacac ctatttgcat tgctgacaag 1020  
 gaatacacga acatcttctt caaatttggg tctggctatg taagtggctg ggcaagagtc 1080  
 ttccacaaag ggagatcagc tttagttctt cagtacctta gagttccact tgttgaccga 1140  
 gccacatgtc ttcgatctac aaagttcacc atctataaca acatgttctg tgctggcttc 1200  
 catgaaggag gtagagattc atgtcaagga gatagtgggg gaccccatgt tactgaagtg 1260  
 gaagggacca gtttcttaac tggaattatt agctggggtg aagagtgtgc aatgaaaggc 1320  
 aaatatggaa tatataccaa ggtatcccg tatgtcaact ggattaagga aaaaacaaag 1380  
 ctcaactaat gaaagatgga tttccaaggt taattcattg gaattgaaaa ttaacag 1437

<210> 10  
 <211> 462  
 <212> PRT  
 <213> Homo sapiens

<400> 10  
 Met Gln Arg Val Asn Met Ile Met Ala Glu Ser Pro Ser Leu Ile Thr  
 1 5 10 15  
 Ile Cys Leu Leu Gly Tyr Leu Leu Ser Ala Glu Cys Thr Val Phe Leu  
 20 25 30  
 Asp His Glu Asn Ala Asn Lys Ile Leu Asn Arg Pro Lys Arg Tyr Asn  
 35 40 45  
 Ser Gly Lys Leu Glu Glu Phe Val Gln Gly Asn Leu Glu Arg Glu Cys

50					55					60					
Met	Glu	Glu	Lys	Cys	Ser	Phe	Glu	Glu	Pro	Arg	Glu	Val	Phe	Glu	Asn
65					70					75					80
Thr	Glu	Lys	Thr	Thr	Glu	Phe	Trp	Lys	Gln	Tyr	Val	Asp	Gly	Asp	Gln
				85					90					95	
Cys	Glu	Ser	Asn	Pro	Cys	Leu	Asn	Gly	Gly	Ser	Cys	Lys	Asp	Asp	Ile
			100					105					110		
Asn	Ser	Tyr	Glu	Cys	Trp	Cys	Pro	Phe	Gly	Phe	Glu	Gly	Lys	Asn	Cys
		115					120					125			
Glu	Leu	Asp	Val	Thr	Cys	Asn	Ile	Lys	Asn	Gly	Arg	Cys	Glu	Gln	Phe
	130					135					140				
Cys	Lys	Asn	Ser	Ala	Asp	Asn	Lys	Val	Val	Cys	Ser	Cys	Thr	Glu	Gly
145					150					155					160
Tyr	Arg	Leu	Ala	Glu	Asn	Gln	Lys	Ser	Cys	Glu	Pro	Ala	Val	Pro	Phe
				165					170					175	
Pro	Cys	Gly	Arg	Val	Ser	Val	Ser	Gln	Thr	Ser	Lys	Leu	Thr	Arg	Ala
			180					185					190		
Glu	Ala	Val	Phe	Pro	Asp	Val	Asp	Tyr	Val	Asn	Pro	Thr	Glu	Ala	Glu
		195					200					205			
Thr	Ile	Leu	Asp	Asn	Ile	Thr	Gln	Gly	Thr	Gln	Ser	Phe	Asn	Asp	Phe
	210					215					220				
Thr	Arg	Val	Val	Gly	Gly	Glu	Asp	Ala	Lys	Pro	Gly	Gln	Phe	Pro	Trp
225					230					235					240
Gln	Val	Val	Leu	Asn	Gly	Lys	Val	Asp	Ala	Phe	Cys	Gly	Gly	Ser	Ile
			245						250					255	
Val	Asn	Glu	Lys	Trp	Ile	Val	Thr	Ala	Ala	His	Cys	Val	Glu	Thr	Gly
		260						265					270		
Val	Lys	Ile	Thr	Val	Val	Ala	Gly	Glu	His	Asn	Ile	Glu	Glu	Thr	Glu
	275					280						285			
His	Thr	Glu	Gln	Lys	Arg	Asn	Val	Ile	Arg	Ala	Ile	Ile	Pro	His	His
	290					295					300				
Asn	Tyr	Asn	Ala	Ala	Ile	Asn	Lys	Tyr	Asn	His	Asp	Ile	Ala	Leu	Leu
305					310					315					320
Glu	Leu	Asp	Glu	Pro	Leu	Val	Leu	Asn	Ser	Tyr	Val	Thr	Pro	Ile	Cys
				325					330					335	
Ile	Ala	Asp	Lys	Glu	Tyr	Thr	Asn	Ile	Phe	Leu	Lys	Phe	Gly	Ser	Gly
		340					345						350		
Tyr	Val	Ser	Gly	Trp	Ala	Arg	Val	Phe	His	Lys	Gly	Arg	Ser	Ala	Leu
		355					360					365			
Val	Leu	Gln	Tyr	Leu	Arg	Val	Pro	Leu	Val	Asp	Arg	Ala	Thr	Cys	Leu
	370					375					380				

Arg Ser Thr Lys Phe Thr Ile Tyr Asn Asn Met Phe Cys Ala Gly Phe  
385 390 395 400

His Glu Gly Gly Arg Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro His  
405 410 415

Val Thr Glu Val Glu Gly Thr Ser Phe Leu Thr Gly Ile Ile Ser Trp  
420 425 430

Gly Glu Glu Cys Ala Met Lys Gly Lys Tyr Gly Ile Tyr Thr Lys Val  
435 440 445

Ser Arg Tyr Val Asn Trp Ile Lys Glu Lys Thr Lys Leu Thr  
450 455 460

<210> 11  
<211> 603  
<212> DNA  
<213> Homo sapiens

<400> 11  
atggattact acagaaaata tgcagctatc tttctgggtca cattgtcggg gtttctgcat 60  
gttctccatt ccgctcctga tgtgcaggat tgcccagaat gcacgctaca ggaaaaccca 120  
ttcttctccc agccgggtgc cccaatactt cagtgcattg gctgctgctt ctctagagca 180  
tatcccactc cactaaggtc caagaagacg atgttggtcc aaaagaacgt cacctcagag 240  
tccacttgct gtgtagctaa atcatataac agggtcacag taatgggggg tttcaaagtg 300  
gagaaccaca cggcgtgcca ctgcagtact tggtattatc acaaattotta aatgttttac 360  
caagtgtgtg cttgatgact gctgattttc tggaatggaa aattaagttg tttagtgttt 420  
atggctttgt gagataaaac tctccttttc cttaccatac cactttgaca cgcttcaagg 480  
atatactgca gctttactgc cttcctcctt atcctacagt acaatcagca gtctagttct 540  
tttcatttgg aatgaataca gcattaagct tgttccactg caaataaagc cttttaaatc 600  
atc 603

<210> 12  
<211> 116  
<212> PRT  
<213> Homo sapiens

<400> 12  
Met Asp Tyr Tyr Arg Lys Tyr Ala Ala Ile Phe Leu Val Thr Leu Ser  
1 5 10 15  
Val Phe Leu His Val Leu His Ser Ala Pro Asp Val Gln Asp Cys Pro  
20 25 30  
Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro  
35 40 45  
Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro  
50 55 60

Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu  
65 70 75 80

Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met Gly  
85 90 95

Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr  
100 105 110

Tyr His Lys Ser  
115

<210> 13  
<211> 390  
<212> DNA  
<213> Homo sapiens

<400> 13  
atgaagacac tccagttttt cttccttttc tgttgctgga aagcaatctg ctgcaatagc 60  
tgtgagctga ccaacatcac cattgcaata gagaaagaag aatgtcggtt ctgcataagc 120  
atcaacacca cttggtgtgc tggctactgc tacaccaggg atctggtgta taaggaccca 180  
gccaggccca aaatccagaa aacatgtacc ttcaaggaaac tggatatatga aacagtgaga 240  
gtgcccggct gtgctcacca tgcagattcc ttgtatacat acccagtggc caccagtgt 300  
cactgtggca agtgtgacag cgacagcact gattgtactg tgcgaggcct ggggcccagc 360  
tactgtcctt ttggtgaaat gaaagaataa 390

<210> 14  
<211> 129  
<212> PRT  
<213> Homo sapiens

<400> 14  
Met Lys Thr Leu Gln Phe Phe Phe Leu Phe Cys Cys Trp Lys Ala Ile  
1 5 10 15  
Cys Cys Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys  
20 25 30  
Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly  
35 40 45  
Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys  
50 55 60  
Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg  
65 70 75 80  
Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val  
85 90 95  
Ala Thr Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys  
100 105 110  
Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu Met Lys

	115	120	125	
Glu				
<210>	15			
<211>	1342			
<212>	DNA			
<213>	Homo sapiens			
<400>	15			
cccggagccg gaccggggcc accgcgcccg ctctgctccg acaccgcgcc ccctggacag				60
ccgccctctc ctccaggccc gtggggctgg ccctgcaccg ccgagcttcc cgggatgagg				120
gcccccggtg tggtcacccg gcgcgcccc a ggtcgtgag ggaccccggc caggcgcgga				180
gatgggggtg cacgaatgtc ctgcctggct gtggcttctc ctgtccctgc tgcgctccc				240
tctgggcctc ccagtcctgg gcgccccacc acgcctcatc tgtgacagcc gagtcctgga				300
gaggtacctc ttggaggcca aggaggccga gaatatcacg acgggctgtg ctgaacactg				360
cagcttgaat gagaatatca ctgtcccaga caccaaagtt aatttctatg cctggaagag				420
gatggaggtc gggcagcagg ccgtagaagt ctggcagggc ctggccctgc tgcggaagc				480
tgtcctgcgg ggccaggccc tgttggtcaa ctcttcccag ccgtgggagc ccctgcagct				540
gcatgtggat aaagccgtca gtggccttcg cagcctcacc actctgcttc gggctctgcg				600
agcccagaag gaagccatct cccctccaga tgcggcctca gctgtccac tccgaacaat				660
cactgctgac actttccgca aactcttccg agtctactcc aatttctctc ggggaaagct				720
gaagctgtac acaggggagg cctgcaggac aggggacaga tgaccagggtg tgtccacctg				780
ggcatatcca ccacctccct caccaacatt gcttgtgcca caccctcccc cgccactcct				840
gaaccccgtc gaggggctct cagctcagcg ccagcctgtc ccatggacac tccagtgcc				900
gcaatgacat ctgaggggcc agaggaactg tccagagagc aactctgaga tctaaggatg				960
tcacagggcc aacttgaggg ccagagcag gaagcattca gagagcagct ttaaaactcag				1020
ggacagagcc atgctgggaa gacgcctgag ctactcggc accctgcaaa atttgatgcc				1080
aggacacgct ttggaggcga ttacctgtt ttgcaccta ccatcaggga caggatgacc				1140
tggagaactt aggtggcaag ctgtgacttc tccaggtctc acgggcatgg gactccctt				1200
ggtggcaaga gcccccttga caccggggtg gtgggaacca tgaagacagg atgggggctg				1260
gcctctggct ctcatggggt ccaagttttg tgtattcttc aacctcattg acaagaactg				1320
aaaccaccaa aaaaaaaaaa aa				1342
<210>	16			
<211>	193			
<212>	PRT			
<213>	Homo sapiens			

&lt;400&gt; 16

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu  
 1 5 10 15

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu  
 20 25 30

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu  
 35 40 45

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu  
 50 55 60

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg  
 65 70 75 80

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu  
 85 90 95

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser  
 100 105 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly  
 115 120 125

Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Arg Ala Gln Lys Glu  
 130 135 140

Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile  
 145 150 155 160

Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu  
 165 170 175

Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp  
 180 185 190

Arg

&lt;210&gt; 17

&lt;211&gt; 435

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 17

atgtggctgc agagcctgct gctcttgggc actgtggcct gcagcatctc tgcacccgcc 60  
 cgctcgccca gcccagcac gcagccctgg gagcatgtga atgccatcca ggaggcccgg 120  
 cgtctcctga acctgagtag agacactgct gctgagatga atgaaacagt agaagtcac 180  
 tcagaaatgt ttgacctoca ggagccgacc tgcctacaga cccgcctgga gctgtacaag 240  
 cagggcctgc ggggcagcct caccaagctc aaggggccct tgaccatgat ggccagccac 300  
 tacaagcagc actgccctcc aaccccgga acttctgtg caaccagat tatcaccttt 360  
 gaaagtttca aagagaacct gaaggacttt ctgcttgtca tcccctttga ctgctgggag 420

ccagtccagg agtga

435

<210> 18  
 <211> 144  
 <212> PRT  
 <213> Homo sapiens

<400> 18  
 Met Trp Leu Gln Ser Leu Leu Leu Leu Gly Thr Val Ala Cys Ser Ile  
 1 5 10 15  
 Ser Ala Pro Ala Arg Ser Pro Ser Pro Ser Thr Gln Pro Trp Glu His  
 20 25 30  
 Val Asn Ala Ile Gln Glu Ala Arg Arg Leu Leu Asn Leu Ser Arg Asp  
 35 40 45  
 Thr Ala Ala Glu Met Asn Glu Thr Val Glu Val Ile Ser Glu Met Phe  
 50 55 60  
 Asp Leu Gln Glu Pro Thr Cys Leu Gln Thr Arg Leu Glu Leu Tyr Lys  
 65 70 75 80  
 Gln Gly Leu Arg Gly Ser Leu Thr Lys Leu Lys Gly Pro Leu Thr Met  
 85 90 95  
 Met Ala Ser His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Thr Ser  
 100 105 110  
 Cys Ala Thr Gln Ile Ile Thr Phe Glu Ser Phe Lys Glu Asn Leu Lys  
 115 120 125  
 Asp Phe Leu Leu Val Ile Pro Phe Asp Cys Trp Glu Pro Val Gln Glu  
 130 135 140

<210> 19  
 <211> 501  
 <212> DNA  
 <213> Homo sapiens

<400> 19  
 atgaaatata caagttatat cttggctttt cagctctgca tcgttttggg ttctcttggc 60  
 tgttactgcc aggaccata tgtaaaagaa gcagaaaacc ttaagaaata ttttaatgca 120  
 ggatcattcag atgtagcgga taatggaact cttttcttag gcattttgaa gaattggaaa 180  
 gaggagagtg acagaaaaat aatgcagagc caaattgtct ctttttactt caaacttttt 240  
 aaaaacttta aagatgacca gagcatccaa aagagtgtgg agaccatcaa ggaagacatg 300  
 aatgtcaagt ttttcaatag caacaaaaag aaacgagatg acttcgaaaa gctgactaat 360  
 tattcggtaa ctgacttgaa tgtccaacgc aaagcaatac atgaactcat ccaagtgatg 420  
 gctgaactgt cgccagcagc taaaacaggg aagcgaaaaa ggagtcagat gctgtttcga 480  
 ggtcgaagag catcccagta a 501

<210> 20  
 <211> 166

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 20

Met Lys Tyr Thr Ser Tyr Ile Leu Ala Phe Gln Leu Cys Ile Val Leu  
 1 5 10 15

Gly Ser Leu Gly Cys Tyr Cys Gln Asp Pro Tyr Val Lys Glu Ala Glu  
 20 25 30

Asn Leu Lys Lys Tyr Phe Asn Ala Gly His Ser Asp Val Ala Asp Asn  
 35 40 45

Gly Thr Leu Phe Leu Gly Ile Leu Lys Asn Trp Lys Glu Glu Ser Asp  
 50 55 60

Arg Lys Ile Met Gln Ser Gln Ile Val Ser Phe Tyr Phe Lys Leu Phe  
 65 70 75 80

Lys Asn Phe Lys Asp Asp Gln Ser Ile Gln Lys Ser Val Glu Thr Ile  
 85 90 95

Lys Glu Asp Met Asn Val Lys Phe Phe Asn Ser Asn Lys Lys Lys Arg  
 100 105 110

Asp Asp Phe Glu Lys Leu Thr Asn Tyr Ser Val Thr Asp Leu Asn Val  
 115 120 125

Gln Arg Lys Ala Ile His Glu Leu Ile Gln Val Met Ala Glu Leu Ser  
 130 135 140

Pro Ala Ala Lys Thr Gly Lys Arg Lys Arg Ser Gln Met Leu Phe Arg  
 145 150 155 160

Gly Arg Arg Ala Ser Gln  
 165

&lt;210&gt; 21

&lt;211&gt; 1352

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 21

ctgggacagt gaatcgacaa tgccgtcttc tgtctcgtgg ggcattcctcc tgctggcagg 60  
 cctgtgctgc ctggtccctg tctccctggc tgaggatccc caggagatg ctgccagaa 120  
 gacagataca tcccaccatg atcaggatca cccaaccttc aacaagatca cccccaacct 180  
 ggctgagttc gccttcagcc tataccgcca gctggcacac cagtccaaca gcaccaatat 240  
 cttcttctcc ccagtgagca tcgctacagc ctttgcaatg ctctccctgg ggaccaaggc 300  
 tgacactcac gatgaaatcc tggagggcct gaatttcaac ctacaggaga ttccggaggc 360  
 tcagatccat gaaggcttcc aggaactcct ccgtaccctc aaccagccag acagccagct 420  
 ccagctgacc accggcaatg gcctgttcct cagcgagggc ctgaagctag tggataagtt 480  
 tttggaggat gttaaaaagt tgtaccactc agaagccttc actgtcaact tcggggacac 540



```

cgaagaggcc aagaaacaga tcaacgatta cgtggagaag ggtactcaag ggaaaattgt 600
ggatttggtc aaggagcttg acagagacac agtttttctg ctggtgaatt acatcttctt 660
taaaggcaaa tgggagagac cctttgaagt caaggacacc gaggaagagg acttccacgt 720
ggaccaggtg accaccgtga aggtgcctat gatgaagcgt ttaggcatgt ttaacatcca 780
gcactgtaag aagctgtcca gctgggtgct gctgatgaaa tacctgggca atgccaccgc 840
catcttcttc ctgcctgatg aggggaaact acagcacctg gaaaatgaac tcacccacga 900
tatcatcacc aagttcctgg aaaatgaaga cagaaggtct gccagcttac atttacccaa 960
actgtccatt actggaacct atgatctgaa gagcgtcctg ggtcaactgg gcatcactaa 1020
ggtcttcagc aatggggctg acctctccgg ggtcacagag gaggcacccc tgaagctctc 1080
caaggccgtg cataaggctg tgctgaccat cgacgagaaa gggactgaag ctgctggggc 1140
catgttttta gaggccatac ccatgtctat ccccccgag gtcaagttca acaaaccctt 1200
tgtcttctta atgattgaac aaaataccaa gtctcccctc ttcattgggaa aagtggtgaa 1260
tcccacccaa aaataactgc ctctcgctcc tcaaccctc cctccatcc ctggccccct 1320
ccctggatga cattaaagaa gggttgagct gg 1352

```

<210> 22  
 <211> 418  
 <212> PRT  
 <213> Homo sapiens

<400> 22  
 Met Pro Ser Ser Val Ser Trp Gly Ile Leu Leu Leu Ala Gly Leu Cys  
 1 5 10 15  
 Cys Leu Val Pro Val Ser Leu Ala Glu Asp Pro Gln Gly Asp Ala Ala  
 20 25 30  
 Gln Lys Thr Asp Thr Ser His His Asp Gln Asp His Pro Thr Phe Asn  
 35 40 45  
 Lys Ile Thr Pro Asn Leu Ala Glu Phe Ala Phe Ser Leu Tyr Arg Gln  
 50 55 60  
 Leu Ala His Gln Ser Asn Ser Thr Asn Ile Phe Phe Ser Pro Val Ser  
 65 70 75 80  
 Ile Ala Thr Ala Phe Ala Met Leu Ser Leu Gly Thr Lys Ala Asp Thr  
 85 90 95  
 His Asp Glu Ile Leu Glu Gly Leu Asn Phe Asn Leu Thr Glu Ile Pro  
 100 105 110  
 Glu Ala Gln Ile His Glu Gly Phe Gln Glu Leu Leu Arg Thr Leu Asn  
 115 120 125  
 Gln Pro Asp Ser Gln Leu Gln Leu Thr Thr Gly Asn Gly Leu Phe Leu  
 130 135 140

Ser Glu Gly Leu Lys Leu Val Asp Lys Phe Leu Glu Asp Val Lys Lys  
 145 150 155 160  
 Leu Tyr His Ser Glu Ala Phe Thr Val Asn Phe Gly Asp Thr Glu Glu  
 165 170 175  
 Ala Lys Lys Gln Ile Asn Asp Tyr Val Glu Lys Gly Thr Gln Gly Lys  
 180 185 190  
 Ile Val Asp Leu Val Lys Glu Leu Asp Arg Asp Thr Val Phe Ala Leu  
 195 200 205  
 Val Asn Tyr Ile Phe Phe Lys Gly Lys Trp Glu Arg Pro Phe Glu Val  
 210 215 220  
 Lys Asp Thr Glu Glu Glu Asp Phe His Val Asp Gln Val Thr Thr Val  
 225 230 235 240  
 Lys Val Pro Met Met Lys Arg Leu Gly Met Phe Asn Ile Gln His Cys  
 245 250 255  
 Lys Lys Leu Ser Ser Trp Val Leu Leu Met Lys Tyr Leu Gly Asn Ala  
 260 265 270  
 Thr Ala Ile Phe Phe Leu Pro Asp Glu Gly Lys Leu Gln His Leu Glu  
 275 280 285  
 Asn Glu Leu Thr His Asp Ile Ile Thr Lys Phe Leu Glu Asn Glu Asp  
 290 295 300  
 Arg Arg Ser Ala Ser Leu His Leu Pro Lys Leu Ser Ile Thr Gly Thr  
 305 310 315 320  
 Tyr Asp Leu Lys Ser Val Leu Gly Gln Leu Gly Ile Thr Lys Val Phe  
 325 330 335  
 Ser Asn Gly Ala Asp Leu Ser Gly Val Thr Glu Glu Ala Pro Leu Lys  
 340 345 350  
 Leu Ser Lys Ala Val His Lys Ala Val Leu Thr Ile Asp Glu Lys Gly  
 355 360 365  
 Thr Glu Ala Ala Gly Ala Met Phe Leu Glu Ala Ile Pro Met Ser Ile  
 370 375 380  
 Pro Pro Glu Val Lys Phe Asn Lys Pro Phe Val Phe Leu Met Ile Glu  
 385 390 395 400  
 Gln Asn Thr Lys Ser Pro Leu Phe Met Gly Lys Val Val Asn Pro Thr  
 405 410 415

Gln Lys

&lt;210&gt; 23

&lt;211&gt; 2004

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 23

gctaacctag tgcctatagc taaggcaggt acctgcatcc ttgtttttgt ttagtggatc

60

ctctatcctt cagagactct ggaacccctg tggctctctc ttcctctaata gaccctgagg 120  
 ggatggagtt ttcaagtcct tccagagagg aatgtcccaa gcctttgagt agggtaagca 180  
 tcatggctgg cagcctcaca ggtttgcttc tacttcaggc agtgctgtgg gcatcagggtg 240  
 cccgcccctg catccctaaa agcttcggct acagctcggg ggtgtgtgtc tgcaatgcc 300  
 catactgtga ctcccttgac ccccgacct ttcctgccct tggtagcttc agccgtatg 360  
 agagtacacg cagtggggca cggatggagc tgagtatggg gccatccag gctaatacaca 420  
 cgggcacagg cctgctactg accctgcagc cagaacagaa gttccagaaa gtgaagggat 480  
 ttggaggggc catgacagat gctgctgtc tcaacatcct tgccctgtca cccctgccc 540  
 aaaatttgct acttaaatcg tacttctctg aagaaggaa cggatataac atcatccggg 600  
 taccatggc cagctgtgac ttctccatcc gcacctacac ctatgcagac accctgatg 660  
 atttccagtt gcacaacttc agcctcccag aggaagatac caagctcaag ataccctga 720  
 ttaccgagc cctgcagttg gccagcgtc cggtttact ccttgccagc ccctggacat 780  
 caccacttg gctcaagacc aatggagcgg tgaatgggaa ggggtcactc aagggacagc 840  
 ccggagacat ctaccaccag acctgggcca gatactttgt gaagttcctg gatgcctatg 900  
 ctgagcaciaa gttacagttc tgggcagtga cagctgaaaa tgagccttct gctgggctgt 960  
 tgagtggata ccccttcag tgcctgggct tcaccctga acatcagcga gacttcattg 1020  
 cccgtgacct aggtcctacc ctgcacaaca gtactacca caatgtccgc ctactcatgc 1080  
 tggatgacca acgcttgctg ctgcccact gggcagaagg ggtactgaca gaccagaag 1140  
 cagctaaata tgttcatggc attgctgtac attggtacct ggactttctg gctccagcca 1200  
 aagccacct aggggagaca caccgcctgt tcccaaacac catgctcttt gcctcagagg 1260  
 cctgtgtggg ctccaagttc tgggagcaga gtgtgaggct aggtcctgg gatcgaggga 1320  
 tgcagtacag ccacagcatc atcacgaacc tcctgtacca tgtggctggc tggaccgact 1380  
 ggaaccttgc cctgaacccc gaaggaggac ccaattgggt gcgtaacttt gtcgacagtc 1440  
 ccatcattgt agacatcac aaggacagct tttaacaaca gccatgttc taccaccttg 1500  
 gccacttcag caagttcatt cctgagggt cccagagagt ggggtgtggt gccagtcaga 1560  
 agaacgacct ggacgcagtg gcactgatgc atccgatgg ctctgctgtt gtggctgtgc 1620  
 taaaccgctc ctctaaggat gtgcctctta ccatcaagga tcctgctgtg ggcttcctgg 1680  
 agacaatctc acctggctac tccattcaca cctacctgtg gcatcgccag tgatggagca 1740  
 gatactcaag gaggcactgg gctcagcctg ggcattaaag ggacagagtc agctcacacg 1800  
 ctgtctgtga ctaaagaggg cacagcaggg ccagtgtgag cttacagcga cgtaagccca 1860  
 ggggcaatgg tttgggtgac tcaactttccc ctctaggtg tgcccagggc tggaggcccc 1920

tagaaaaaga tcagtaagcc ccagtgtccc cccagccccc atgcttatgt gaacatgcgc 1980

tgtgtgctgc ttgctttgga aact 2004

<210> 24  
 <211> 536  
 <212> PRT  
 <213> Homo sapiens

<400> 24  
 Met Glu Phe Ser Ser Pro Ser Arg Glu Glu Cys Pro Lys Pro Leu Ser  
 1 5 10 15  
 Arg Val Ser Ile Met Ala Gly Ser Leu Thr Gly Leu Leu Leu Leu Gln  
 20 25 30  
 Ala Val Ser Trp Ala Ser Gly Ala Arg Pro Cys Ile Pro Lys Ser Phe  
 35 40 45  
 Gly Tyr Ser Ser Val Val Cys Val Cys Asn Ala Thr Tyr Cys Asp Ser  
 50 55 60  
 Phe Asp Pro Pro Thr Phe Pro Ala Leu Gly Thr Phe Ser Arg Tyr Glu  
 65 70 75 80  
 Ser Thr Arg Ser Gly Arg Arg Met Glu Leu Ser Met Gly Pro Ile Gln  
 85 90 95  
 Ala Asn His Thr Gly Thr Gly Leu Leu Leu Thr Leu Gln Pro Glu Gln  
 100 105 110  
 Lys Phe Gln Lys Val Lys Gly Phe Gly Gly Ala Met Thr Asp Ala Ala  
 115 120 125  
 Ala Leu Asn Ile Leu Ala Leu Ser Pro Pro Ala Gln Asn Leu Leu Leu  
 130 135 140  
 Lys Ser Tyr Phe Ser Glu Glu Gly Ile Gly Tyr Asn Ile Ile Arg Val  
 145 150 155 160  
 Pro Met Ala Ser Cys Asp Phe Ser Ile Arg Thr Tyr Thr Tyr Ala Asp  
 165 170 175  
 Thr Pro Asp Asp Phe Gln Leu His Asn Phe Ser Leu Pro Glu Glu Asp  
 180 185 190  
 Thr Lys Leu Lys Ile Pro Leu Ile His Arg Ala Leu Gln Leu Ala Gln  
 195 200 205  
 Arg Pro Val Ser Leu Leu Ala Ser Pro Trp Thr Ser Pro Thr Trp Leu  
 210 215 220  
 Lys Thr Asn Gly Ala Val Asn Gly Lys Gly Ser Leu Lys Gly Gln Pro  
 225 230 235 240  
 Gly Asp Ile Tyr His Gln Thr Trp Ala Arg Tyr Phe Val Lys Phe Leu  
 245 250 255  
 Asp Ala Tyr Ala Glu His Lys Leu Gln Phe Trp Ala Val Thr Ala Glu  
 260 265 270

Asn Glu Pro Ser Ala Gly Leu Leu Ser Gly Tyr Pro Phe Gln Cys Leu  
 275 280 285  
 Gly Phe Thr Pro Glu His Gln Arg Asp Phe Ile Ala Arg Asp Leu Gly  
 290 295 300  
 Pro Thr Leu Ala Asn Ser Thr His His Asn Val Arg Leu Leu Met Leu  
 305 310 315 320  
 Asp Asp Gln Arg Leu Leu Leu Pro His Trp Ala Lys Val Val Leu Thr  
 325 330 335  
 Asp Pro Glu Ala Ala Lys Tyr Val His Gly Ile Ala Val His Trp Tyr  
 340 345 350  
 Leu Asp Phe Leu Ala Pro Ala Lys Ala Thr Leu Gly Glu Thr His Arg  
 355 360 365  
 Leu Phe Pro Asn Thr Met Leu Phe Ala Ser Glu Ala Cys Val Gly Ser  
 370 375 380  
 Lys Phe Trp Glu Gln Ser Val Arg Leu Gly Ser Trp Asp Arg Gly Met  
 385 390 395 400  
 Gln Tyr Ser His Ser Ile Ile Thr Asn Leu Leu Tyr His Val Val Gly  
 405 410 415  
 Trp Thr Asp Trp Asn Leu Ala Leu Asn Pro Glu Gly Gly Pro Asn Trp  
 420 425 430  
 Val Arg Asn Phe Val Asp Ser Pro Ile Ile Val Asp Ile Thr Lys Asp  
 435 440 445  
 Thr Phe Tyr Lys Gln Pro Met Phe Tyr His Leu Gly His Phe Ser Lys  
 450 455 460  
 Phe Ile Pro Glu Gly Ser Gln Arg Val Gly Leu Val Ala Ser Gln Lys  
 465 470 475 480  
 Asn Asp Leu Asp Ala Val Ala Leu Met His Pro Asp Gly Ser Ala Val  
 485 490 495  
 Val Val Val Leu Asn Arg Ser Ser Lys Asp Val Pro Leu Thr Ile Lys  
 500 505 510  
 Asp Pro Ala Val Gly Phe Leu Glu Thr Ile Ser Pro Gly Tyr Ser Ile  
 515 520 525  
 His Thr Tyr Leu Trp His Arg Gln  
 530 535

&lt;210&gt; 25

&lt;211&gt; 1726

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 25

atggatgcaa tgaagagagg gctctgctgt gtgctgctgc tgtgtggagc agtcttcgtt 60

tcgcccagcc aggaaatcca tgcccgattc agaagaggag ccagatctta ccaagtgatc 120

tgcagagatg aaaaaacgca gatgatatac cagcaacatc agtcatggct gcgccctgtg 180  
 ctcagaagca accgggtgga atattgctgg tgcaacagtg gcagggcaca gtgccactca 240  
 gtgcctgtca aaagttgcag cgagccaagg tgtttcaacg ggggcacctg ccagcaggcc 300  
 ctgtacttct cagatttcgt gtgccagtgc cccgaaggat ttgctgggaa gtgctgtgaa 360  
 atagatacca gggccacgtg ctacgaggac cagggcatca gctacagggg cacgtggagc 420  
 acagcggaga gtggcgccga gtgcaccaac tggaacagca gcgcgttggc ccagaagccc 480  
 tacagcgggc ggaggccaga cgccatcagg ctgggcctgg ggaaccacaa ctactgcaga 540  
 aaccagatc gagactcaaa gccctggtgc tacgtcttta aggcggggaa gtacagctca 600  
 gagttctgca gcacccctgc ctgctctgag ggaaacagtg actgctactt tgggaatggg 660  
 tcagcctacc gtggcacgca cagcctcacc gagtccgggtg cctcctgcct cccgtggaat 720  
 tccatgatcc tgataggcaa ggtttacaca gcacagaacc ccagtgccca ggcactgggc 780  
 ctgggcaaac ataattactg ccggaatcct gatggggatg ccaagccctg gtgccacgtg 840  
 ctgaagaacc gcaggctgac gtgggagtag tgtgatgtgc cctcctgctc cacctgcggc 900  
 ctgagacagt acagccagcc tcagtttcgc atcāaaggag ggctcttcgc cgacatcgcc 960  
 tcccaccctt ggcaggctgc catctttgcc aagcacagga ggtcgcggg agagcggttc 1020  
 ctgtgcgggg gcatactcat cagctcctgc tggattctct ctgccgcca ctgcttcag 1080  
 gagaggtttc cgccccacca cctgacgggtg atcttgggca gaacataccg ggtggtccct 1140  
 ggcgaggagg agcagaaatt tgaagtcgaa aaatacattg tccataagga attcgatgat 1200  
 gacacttacg acaatgacat tgcgctgctg cagctgaaat cggattcgtc ccgctgtgcc 1260  
 caggagagca gcgtggtccg cactgtgtgc cttcccccg cgacactgca gctgccggac 1320  
 tggacggagt gtgagctctc cggctacggc aagcatgagg ccttgtctcc tttctattcg 1380  
 gagcggtga aggaggctca tgtcagactg taccatcca gccgctgac atcacaacat 1440  
 ttacttaaca gaacagtcac cgacaacatg ctgtgtgtg gagacactcg gagcggcggg 1500  
 cccagggcaa acttgacga cgctgccag ggcgattcgg gaggccccct ggtgtgtctg 1560  
 aacgatggcc gcatgacttt ggtgggcatc atcagctggg gcctgggctg tggacagaag 1620  
 gatgtcccgg gtgtgtacac caaggttacc aactacctag actggattcg tgacaacatg 1680  
 cgaccgtgac caggaacacc cgactcctca aaagcaaatg agatcc 1726

<210> 26  
 <211> 562  
 <212> PRT  
 <213> Homo sapiens

<400> 26

Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu Leu Cys Gly  
 1 5 10 15  
 Ala Val Phe Val Ser Pro Ser Gln Glu Ile His Ala Arg Phe Arg Arg  
 20 25 30  
 Gly Ala Arg Ser Tyr Gln Val Ile Cys Arg Asp Glu Lys Thr Gln Met  
 35 40 45  
 Ile Tyr Gln Gln His Gln Ser Trp Leu Arg Pro Val Leu Arg Ser Asn  
 50 55 60  
 Arg Val Glu Tyr Cys Trp Cys Asn Ser Gly Arg Ala Gln Cys His Ser  
 65 70 75 80  
 Val Pro Val Lys Ser Cys Ser Glu Pro Arg Cys Phe Asn Gly Gly Thr  
 85 90 95  
 Cys Gln Gln Ala Leu Tyr Phe Ser Asp Phe Val Cys Gln Cys Pro Glu  
 100 105 110  
 Gly Phe Ala Gly Lys Cys Cys Glu Ile Asp Thr Arg Ala Thr Cys Tyr  
 115 120 125  
 Glu Asp Gln Gly Ile Ser Tyr Arg Gly Thr Trp Ser Thr Ala Glu Ser  
 130 135 140  
 Gly Ala Glu Cys Thr Asn Trp Asn Ser Ser Ala Leu Ala Gln Lys Pro  
 145 150 155 160  
 Tyr Ser Gly Arg Arg Pro Asp Ala Ile Arg Leu Gly Leu Gly Asn His  
 165 170 175  
 Asn Tyr Cys Arg Asn Pro Asp Arg Asp Ser Lys Pro Trp Cys Tyr Val  
 180 185 190  
 Phe Lys Ala Gly Lys Tyr Ser Ser Glu Phe Cys Ser Thr Pro Ala Cys  
 195 200 205  
 Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr Arg  
 210 215 220  
 Gly Thr His Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp Asn  
 225 230 235 240  
 Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala  
 245 250 255  
 Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly  
 260 265 270  
 Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp  
 275 280 285  
 Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr  
 290 295 300  
 Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala  
 305 310 315 320  
 Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser Pro

Arg Pro

<210> 27

<211> 825

<212> DNA

<213> Homo sapiens

<400> 27.

atcactctct ttaatcacta ctcacattaa cctcaactcc tgccacaatg tacaggatgc 60

aactcctgtc ttgcattgca ctaattcttg cacttgtcac aaacagtgca cctacttcaa 120

gttcgacaaa gaaaacaaag aaaacacagc tacaactgga gcatttactg ctggatttac 180

agatgatttt gaatggaatt aataattaca agaatcccaa actcaccagg atgctcacat 240

ttaagtttta catgcccaag aaggccacag aactgaaaca gcttcagtgt ctagaagaag 300



aactcaaacc tctggaggaa gtgctgaatt tagctcaaag caaaaacttt cacttaagac 360  
ccagggactt aatcagcaat atcaacgtaa tagttctgga actaaaggga tctgaaacaa 420  
cattcatgtg tgaatatgca gatgagacag caaccattgt agaatttctg aacagatgga 480  
ttaccttttg tcaaagcatc atctcaacac taacttgata attaagtgtc tcccacttaa 540  
aacatatcag gccttctatt tatttattta aatattttaa ttttatattt attgttgaat 600  
gtatggttgc tacctattgt aactattatt cttaatctta aaactataaa tatggatctt 660  
ttatgattct ttttgtaagc cctaggggct ctaaaatggt ttaccttatt tatcccaaaa 720  
atatattatta ttatgttgaa tgttaaatat agtatctatg tagattgggt agtaaaacta 780  
tttaataaat ttgataaata taaaaaaaaa aaacaaaaaa aaaaa 825

<210> 28  
<211> 156  
<212> PRT  
<213> Homo sapiens

<400> 28  
Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ile Leu Ala Leu  
1 5 10 15  
Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Lys Lys  
20 25 30  
Thr Gln Leu Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu  
35 40 45  
Asn Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr  
50 55 60  
Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys Gln Leu Gln  
65 70 75 80  
Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala  
85 90 95  
Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile  
100 105 110  
Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys  
115 120 125  
Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp  
130 135 140  
Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr  
145 150 155

<210> 29  
<211> 7931  
<212> DNA  
<213> Homo sapiens

<400> 29

atgcaaatag agctctccac ctgcttcttt ctgtgccttt tgcgattctg ctttagtgcc 60  
 accagaagat actacctggg tgcagtggaa ctgtcatggg actatatgca aagtgatctc 120  
 ggtgagctgc ctgtggacgc aagatttcct cctagagtgc caaaatcttt tccattcaac 180  
 acctcagtcg tgtacaaaaa gactctgttt gtagaattca cggatcacct tttcaacatc 240  
 gctaagccaa ggccaccctg gatgggtctg ctaggtccta ccatccaggc tgaggtttat 300  
 gatacagtgg tcattacact taagaacatg gcttcccatc ctgtcagctc tcatgctgtt 360  
 ggtgtatcct actggaaagc ttctgagggg gctgaatatg atgatcagac cagtcaaagg 420  
 gagaaagaag atgataaagt cttccctggg ggaagccata catatgtctg gcaggctctg 480  
 aaagagaatg gtccaatggc ctctgaccca ctgtgcctta cctactcata tctttctcat 540  
 gtggacctgg taaaagactt gaattcaggc ctcatggag ccctactagt atgtagagaa 600  
 gggagtctgg ccaaggaaaa gacacagacc ttgcacaaat ttatactact ttttgctgta 660  
 tttgatgaag ggaaaagttg gcactcagaa acaaagaact ccttgatgca ggatagggat 720  
 gctgcactcg ctcgggcctg gcctaaaatg cacacagtca atggttatgt aaacaggctc 780  
 ctgccaggtc tgattggatg ccacaggaaa tcagtctatt ggcatgtgat tggaatgggc 840  
 accactcctg aagtgcactc aatattcctc gaaggtcaca ctttcttgt gaggaacct 900  
 cgccaggcgt ccttggaat ctcgccaata actttcctta ctgctcaaac actcttgatg 960  
 gaccttgac agtttctact gttttgtcat atctcttccc accaacaatga tggcatggaa 1020  
 gcttatgtca aagtagacag ctgtccagag gaacccaac tacgaatgaa aaataatgaa 1080  
 gaagcggaag actatgatga tgatcttact gattctgaaa tggatgtggt caggtttgat 1140  
 gatgacaact ctcttcctt tatccaaatt cgtcaggtg ccaagaagca tcctaaaact 1200  
 tgggtacatt acattgctgc tgaagaggag gactgggact atgctccctt agtcctcgcc 1260  
 cccgatgaca gaagttataa aagtcaatat ttgaacaatg gccctcagcg gattggtagg 1320  
 aagtacaaaa aagtccgatt tatggcatac acagatgaaa ctttaagac tcgtgaagct 1380  
 attcagcatg aatcaggaat cttgggacct ttactttatg gggaagttgg agacacactg 1440  
 ttgattatat ttaagaatca agcaagcaga ccatataaca tctaccctca cggaatcact 1500  
 gatgtccgtc ctttgtatc aaggagatta ccaaaagggtg taaaacatct gaaggatctt 1560  
 ccaattctgc caggagaaat attcaaatat aaatggacag tgactgtaga agatgggcca 1620  
 actaaatcag atcctcgggtg cctgaccgcg tattactcta gtttcgttaa tatggagaga 1680  
 gatctagctt caggactcat tggccctctc ctcatctgct acaaagaatc tgtagatcaa 1740  
 agaggaaaacc agataatgtc agacaagagg aatgtcatcc tgttttctgt atttgatgag 1800  
 aaccgaagct ggtacctcac agagaatata caacgcttct tcccaatcc agctggagtg 1860

cagcttgagg atccagagtt ccaagcctcc aacatcatgc acagcatcaa tggctatggt 1920  
 tttgatagtt tgcagttgtc agtttgtttg catgaggtgg catactggta cattotaagc 1980  
 attggagcac agactgactt cctttctgtc ttcttctctg gatatacctt caaacacaaa 2040  
 atggtctatg aagacacact caccctattc ccattctcag gagaaactgt cttcatgtcg 2100  
 atgaaaacc caggtctatg gattctgggg tgccacaact cagactttcg gaacagaggc 2160  
 atgaccgcct tactgaaggt ttctagtgtg gacaagaaca ctggtgatta ttacgaggac 2220  
 agttatgaag atatttcagc atacttctg agtaaaaaca atgccattga accaagaagc 2280  
 ttotcccaga attcaagaca ccgtagcact aggcaaaagc aatttaatgc caccacaatt 2340  
 ccgaaaatg acatagagaa gactgaccct tggtttgac acagaacacc tatgcctaaa 2400  
 atacaaaatg tctcctctag tgatttggtg atgctcttgc gacagagtcc tactccacat 2460  
 gggctatcct tatctgatct ccaagaagcc aaatatgaga ctttttctga tgatccatca 2520  
 cctggagcaa tagacagtaa taacagcctg tctgaaatga cacacttcag gccacagctc 2580  
 catcacagtg gggacatggt atttaccctt ggtcaggcc tccaattaag attaaatgag 2640  
 aaactgggga caactgcagc aacagagttg aagaaacttg atttcaaagt ttctagtaca 2700  
 tcaaataatc tgatttcaac aattccatca gacaatttgg cagcaggtac tgataataca 2760  
 agttccttag gacccccaaag tatgccagtt cattatgata gtcaattaga taccactcta 2820  
 tttggcaaaa agtcatctcc ccttactgag tctggtggac ctctgagctt gagtgaagaa 2880  
 aataatgatt caaagttggt agaatcaggt ttaatgaata gccaaagaaag ttcatgggga 2940  
 aaaaatgtat cgtcaacaga gagtggtagg ttatttaaag ggaaaagagc tcatggacct 3000  
 gctttgttga ctaaagataa tgccttatcc aaagttagca tctctttgtt aaagacaaac 3060  
 aaaacttcca ataattcagc aactaataga aagactcaca ttgatggccc atcattatta 3120  
 attgagaata gtccatcagt ctggcaaaat atattagaaa gtgacactga gtttaaaaaa 3180  
 gtgacacctt tgattcatga cagaatgctt atggacaaaa atgctacagc tttgaggcta 3240  
 aatcatatgt caaataaaac tacttcatca aaaaacatgg aaatggtcca acagaaaaaa 3300  
 gagggcccca ttccaccaga tgcacaaaat ccagatatgt cgttctttaa gatgctattc 3360  
 ttgccagaat cagcaaggtg gatacaaagg actcatggaa agaactctct gaactctggg 3420  
 caaggcccca gtccaaagca attagtatcc ttaggaccag aaaaatctgt ggaaggtcag 3480  
 aatttcttgt ctgagaaaaa caaagtggta gtaggaaagg gtgaatttac aaaggacgta 3540  
 ggactcaaag agatgggttt tccaagcagc agaaacctat ttcttactaa cttggataat 3600  
 ttacatgaaa ataatacaca caatcaagaa aaaaaaatc aggaagaaat agaaaagaag 3660

gaaacattaa tccaagagaa tgtagttttg cctcagatac atacagtac tggcactaag 3720  
aatttcatga agaacctttt cttactgagc actaggcaaa atgtagaagg ttcatatgac 3780  
ggggcatatg ctccagtact tcaagatttt aggtcattaa atgattcaac aaatagaaca 3840  
aagaaacaca cagctcattt ctcaaaaaa ggggaggaag aaaacttgga aggcttggga 3900  
aatcaaacca agcaaattgt agagaaatat gcatgcacca caaggatatc tcctaataca 3960  
agccagcaga attttgtcac gcaacgtagt aagagagctt tgaacaatt cagactccca 4020  
ctagaagaaa cagaacttga aaaaaggata attgtggatg acacctcaac ccagtgggtcc 4080  
aaaaacatga aacatttgac cccgagcacc ctcacacaga tagactacaa tgagaaggag 4140  
aaaggggcca ttactcagtc tcccttatca gattgcctta cgaggagtca tagcatccct 4200  
caagcaaata gatctccatt acccattgca aaggatcat catttccatc tattagacct 4260  
atatatctga ccagggtcct attccaagac aactcttctc atcttcagc agcatcttat 4320  
agaaagaaag attctggggt ccaagaaagc agtcatttct tacaaggagc caaaaaaat 4380  
aacctttctt tagccattct aaccttggag atgactggtg atcaaagaga ggttgggtcc 4440  
ctggggacaa gtgccacaaa ttcagtcaca tacaagaaag ttgagaacac tgttctcccg 4500  
aaaccagact tgcccaaac atctggcaaa gttgaattgc ttccaaaagt tcacatttat 4560  
cagaaggacc tattccctac ggaaactagc aatgggtctc ctggccatct ggatctcgtg 4620  
gaaggagacc ttcttcaggg aacagaggga gcgattaagt ggaatgaagc aaacagacct 4680  
ggaaaagttc cttttctgag agtagcaaca gaaagctctg caaagactcc ctccaagcta 4740  
ttggatcctc ttgcttggga taaccactat ggtaetcaga taccaaaaga agagtggaaa 4800  
tccaagaga agtcaccaga aaaaacagct tttaagaaaa aggataccat tttgtccctg 4860  
aacgcttggtg aaagcaatca tgcaatagca gcaataaatg agggacaaaa taagcccgaa 4920  
atagaagtca cctgggcaaa gcaaggtagg actgaaaggc tgtgctctca aaaccaccca 4980  
gtcttgaaac gccatcaacg ggaaataact cgtactactc ttcagtcaga tcaagaggaa 5040  
attgactatg atgataccat atcagttgaa atgaagaagg aagattttga catttatgat 5100  
gaggatgaaa atcagagccc ccgcagcttt caaaagaaaa cacgacacta ttttattgct 5160  
gcagtggaga ggtctggga ttatgggatg agtagctccc cacatgttct aagaaacagg 5220  
gtcagagtgt gcagtgtccc tcagttcaag aaagttgttt tcaggaatt tactgatggc 5280  
tcctttactc agcccttata ccgtggagaa cttaatgaac atttgggact cctggggcca 5340  
tatataagag cagaagttga agataatatc atggtaactt tcagaaatca ggctctcgt 5400  
ccctattcct tctattctag ccttatttct tatgaggaag atcagaggca aggagcagaa 5460  
cctagaaaaa actttgtcaa gcctaataa accaaaactt acttttggaa agtgcaacat 5520

catatggcac ccactaaaga tgagtttgac tgcaaagcct gggcttattt ctctgatgtt 5580  
 gacctggaaa aagatgtgca ctgaggcctg attggacccc ttctgggtctg ccacactaac 5640  
 aactgaacc ctgctcatgg gagacaagtg acagtacagg aatttgctct gtttttcacc 5700  
 atctttgatg agacaaaag ctggtacttc actgaaaata tggaaagaaa ctgcagggt 5760  
 ccttgcaata tccagatgga agatcccact tttaaagaga attatcgctt ccatgcaatc 5820  
 aatggctaca taatggatac actacctggc ttagtaatgg ctgaggatca aaggattcga 5880  
 tggatatctg tcagcatggg cagcaatgaa aacatccatt ctattcattt cagtggacat 5940  
 gtgttcactg tacgaaaaaa agaggagtat aaaatggcac tgtacaatct ctatccaggt 6000  
 gtttttgaga cagtggaaat gttaccatcc aaagctggaa tttggcgggt ggaatgcctt 6060  
 attggcgagc atctacatgc tgggatgagc acactttttc tgggtgtacag caataagtgt 6120  
 cagactcccc tgggaatggc ttctggacac attagagatt ttcagattac agcttcagga 6180  
 caatatggac agtgggcccc aaagctggc agacttcatt attccggatc aatcaatgcc 6240  
 tggagcacca aggagccctt ttcttgatc aaggtggatc tgttggcacc aatgattatt 6300  
 caggcatca agaccagggt tgcccgtcag aagttctoca gcctctacat ctctcagttt 6360  
 atcatcatgt atagtcttga tgggaagaag tggcagactt atcgaggaaa ttccactgga 6420  
 acctaatgg tcttctttgg caatgtggat tcatctggga taaaacacaa tatttttaac 6480  
 cctccaatta ttgctcgata catccgtttg caccacaactc attatagcat tcgcagcact 6540  
 ctctgcatgg agttgatggg ctgtgattta aatagttgca gcatgccatt gggaatggag 6600  
 agtaaagcaa tatcagatgc acagattact gcttcacct actttacca tatgtttgcc 6660  
 acctgggtctc cttcaaaagc tcgacttcac ctccaaggga ggagtaatgc ctggagacct 6720  
 caggtgaata atccaaaaga gtggctgcaa gtggacttcc agaagacaat gaaagtcaca 6780  
 ggagtaacta ctgaggaggt aaaatctctg cttaccagca tgtatgtgaa ggagttcctc 6840  
 atctccagca gtcaagatgg ccatcagtggt actctctttt ttcagaatgg caaagtaaag 6900  
 gtttttcagg gaaatcaaga ctccctcaca cctgtggtga actctctaga ccacacgtta 6960  
 ctgactcgct accttcgaat tcaccccag agttgggtgc accagattgc cctgaggatg 7020  
 gaggttctgg gctgcgaggc acaggacctc tactgaggggt ggccactgca gcacctgcca 7080  
 ctgccgtcac ctctccctcc tcagctccag ggcagtgtcc ctccctggct tgccttctac 7140  
 ctttgtgcta aatcctagca gacactgct tgaagcctcc tgaattaaact atcatcagtc 7200  
 ctgcatttct ttggtggggg gccaggagggt tgcaccaat ttaacttaac tcttacctat 7260  
 tttctgcagc tgcctccaga ttactccttc cttccaatat aactaggcaa aaagaagtga 7320

ggagaaacct gcatgaaagc attcttccct gaaaagttag gcctctcaga gtcaccactt 7380  
 cctctgttgt agaaaaacta tgtgatgaaa ctttgaaaaa gatatttatg atgttaacat 7440  
 ttcagggttaa gcctcatagc tttaaaataa aactctcagt tgtttattat cctgatcaag 7500  
 catggaacaa agcatgtttc aggatcagat caatacaatc ttggagtcaa aaggcaaatc 7560  
 atttggacaa tctgcaaaat ggagagaata caataactac tacagtaaag tctgtttctg 7620  
 ctctcttaca catagatata attatgttat ttagtcatta tgagggggcac attcttatct 7680  
 ccaaaactag cattcttaaa ctgagaatta tagatggggt tcaagaatcc ctaagtcccc 7740  
 tgaaattata taaggcattc tgtataaatg caaatgtgca tttttctgac gagtgtccat 7800  
 agatataaag ccatttggtc ttaattctga ccaataaaaa aataagtcag gaggatgcaa 7860  
 ttgttgaaag ctttgaaata aaataacaat gtcttcttga aatttgtgat ggccaagaaa 7920  
 gaaaatgatg a 7931

<210> 30  
 <211> 2351  
 <212> PRT  
 <213> Homo sapiens

<400> 30  
 Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe  
 1 5 10 15  
 Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser  
 20 25 30  
 Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg  
 35 40 45  
 Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
 50 55 60  
 Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
 65 70 75 80  
 Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln  
 85 90 95  
 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
 100 105 110  
 His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser  
 115 120 125  
 Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp  
 130 135 140  
 Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu  
 145 150 155 160  
 Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser  
 165 170 175

Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile  
 180 185 190  
 Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr  
 195 200 205  
 Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
 210 215 220  
 Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp  
 225 230 235 240  
 Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr  
 245 250 255  
 Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val  
 260 265 270  
 Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
 275 280 285  
 Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser  
 290 295 300  
 Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met  
 305 310 315 320  
 Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His  
 325 330 335  
 Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro  
 340 345 350  
 Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp  
 355 360 365  
 Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser  
 370 375 380  
 Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr  
 385 390 395 400  
 Trp Val His Tyr Ile Ala Ala Glu Glu Asp Trp Asp Tyr Ala Pro  
 405 410 415  
 Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn  
 420 425 430  
 Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met  
 435 440 445  
 Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu  
 450 455 460  
 Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu  
 465 470 475 480  
 Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro  
 485 490 495  
 His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys

500										505					510				
Gly	Val	Lys	His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe				
	515						520					525							
Lys	Tyr	Lys	Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp				
	530					535					540								
Pro	Arg	Cys	Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg				
545					550					555					560				
Asp	Leu	Ala	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu				
				565					570					575					
Ser	Val	Asp	Gln	Arg	Gly	Asn	Gln	Ile	Met	Ser	Asp	Lys	Arg	Asn	Val				
			580					585					590						
Ile	Leu	Phe	Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu				
	595						600					605							
Asn	Ile	Gln	Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp				
	610					615					620								
Pro	Glu	Phe	Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val				
625					630					635					640				
Phe	Asp	Ser	Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp				
				645				650						655					
Tyr	Ile	Leu	Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe				
			660					665						670					
Ser	Gly	Tyr	Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr				
		675					680					685							
Leu	Phe	Pro	Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro				
	690					695					700								
Gly	Leu	Trp	Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly				
705					710					715					720				
Met	Thr	Ala	Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp				
				725					730					735					
Tyr	Tyr	Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys				
		740					745							750					
Asn	Asn	Ala	Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	Arg				
		755					760					765							
Ser	Thr	Arg	Gln	Lys	Gln	Phe	Asn	Ala	Thr	Thr	Ile	Pro	Glu	Asn	Asp				
		770				775					780								
Ile	Glu	Lys	Thr	Asp	Pro	Trp	Phe	Ala	His	Arg	Thr	Pro	Met	Pro	Lys				
785					790					795					800				
Ile	Gln	Asn	Val	Ser	Ser	Ser	Asp	Leu	Leu	Met	Leu	Leu	Arg	Gln	Ser				
				805				810						815					
Pro	Thr	Pro	His	Gly	Leu	Ser	Leu	Ser	Asp	Leu	Gln	Glu	Ala	Lys	Tyr				
			820					825					830						



Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn  
 835 840 845  
 Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly  
 850 855 860  
 Asp Met Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu  
 865 870 875 880  
 Lys Leu Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys  
 885 890 895  
 Val Ser Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn  
 900 905 910  
 Leu Ala Ala Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met  
 915 920 925  
 Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys  
 930 935 940  
 Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu  
 945 950 955 960  
 Asn Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu  
 965 970 975  
 Ser Ser Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe  
 980 985 990  
 Lys Gly Lys Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala  
 995 1000 1005  
 Leu Phe Lys Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser  
 1010 1015 1020  
 Asn Asn Ser Ala Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser  
 1025 1030 1035  
 Leu Leu Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu  
 1040 1045 1050  
 Ser Asp Thr Glu Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg  
 1055 1060 1065  
 Met Leu Met Asp Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met  
 1070 1075 1080  
 Ser Asn Lys Thr Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln  
 1085 1090 1095  
 Lys Lys Glu Gly Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met  
 1100 1105 1110  
 Ser Phe Phe Lys Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile  
 1115 1120 1125  
 Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro  
 1130 1135 1140

Ser Pro Lys Gln Leu Val	Ser Leu Gly Pro Glu Lys	Ser Val Glu
1145	1150	1155
Gly Gln Asn Phe Leu Ser	Glu Lys Asn Lys Val Val	Val Gly Lys
1160	1165	1170
Gly Glu Phe Thr Lys Asp	Val Gly Leu Lys Glu Met	Val Phe Pro
1175	1180	1185
Ser Ser Arg Asn Leu Phe	Leu Thr Asn Leu Asp Asn	Leu His Glu
1190	1195	1200
Asn Asn Thr His Asn Gln	Glu Lys Lys Ile Gln Glu	Glu Ile Glu
1205	1210	1215
Lys Lys Glu Thr Leu Ile	Gln Glu Asn Val Val Leu	Pro Gln Ile
1220	1225	1230
His Thr Val Thr Gly Thr	Lys Asn Phe Met Lys Asn	Leu Phe Leu
1235	1240	1245
Leu Ser Thr Arg Gln Asn	Val Glu Gly Ser Tyr Asp	Gly Ala Tyr
1250	1255	1260
Ala Pro Val Leu Gln Asp	Phe Arg Ser Leu Asn Asp	Ser Thr Asn
1265	1270	1275
Arg Thr Lys Lys His Thr	Ala His Phe Ser Lys Lys	Gly Glu Glu
1280	1285	1290
Glu Asn Leu Glu Gly Leu	Gly Asn Gln Thr Lys Gln	Ile Val Glu
1295	1300	1305
Lys Tyr Ala Cys Thr Thr	Arg Ile Ser Pro Asn Thr	Ser Gln Gln
1310	1315	1320
Asn Phe Val Thr Gln Arg	Ser Lys Arg Ala Leu Lys	Gln Phe Arg
1325	1330	1335
Leu Pro Leu Glu Glu Thr	Glu Leu Glu Lys Arg Ile	Ile Val Asp
1340	1345	1350
Asp Thr Ser Thr Gln Trp	Ser Lys Asn Met Lys His	Leu Thr Pro
1355	1360	1365
Ser Thr Leu Thr Gln Ile	Asp Tyr Asn Glu Lys Glu	Lys Gly Ala
1370	1375	1380
Ile Thr Gln Ser Pro Leu	Ser Asp Cys Leu Thr Arg	Ser His Ser
1385	1390	1395
Ile Pro Gln Ala Asn Arg	Ser Pro Leu Pro Ile Ala	Lys Val Ser
1400	1405	1410
Ser Phe Pro Ser Ile Arg	Pro Ile Tyr Leu Thr Arg	Val Leu Phe
1415	1420	1425
Gln Asp Asn Ser Ser His	Leu Pro Ala Ala Ser Tyr	Arg Lys Lys
1430	1435	1440
Asp Ser Gly Val Gln Glu	Ser Ser His Phe Leu Gln	Gly Ala Lys

1445	1450	1455
Lys Asn Asn Leu Ser Leu	Ala Ile Leu Thr Leu	Glu Met Thr Gly
1460	1465	1470
Asp Gln Arg Glu Val Gly	Ser Leu Gly Thr Ser	Ala Thr Asn Ser
1475	1480	1485
Val Thr Tyr Lys Lys Val	Glu Asn Thr Val Leu	Pro Lys Pro Asp
1490	1495	1500
Leu Pro Lys Thr Ser Gly	Lys Val Glu Leu Leu	Pro Lys Val His
1505	1510	1515
Ile Tyr Gln Lys Asp Leu	Phe Pro Thr Glu Thr	Ser Asn Gly Ser
1520	1525	1530
Pro Gly His Leu Asp Leu	Val Glu Gly Ser Leu	Leu Gln Gly Thr
1535	1540	1545
Glu Gly Ala Ile Lys Trp	Asn Glu Ala Asn Arg	Pro Gly Lys Val
1550	1555	1560
Pro Phe Leu Arg Val Ala	Thr Glu Ser Ser Ala	Lys Thr Pro Ser
1565	1570	1575
Lys Leu Leu Asp Pro Leu	Ala Trp Asp Asn His	Tyr Gly Thr Gln
1580	1585	1590
Ile Pro Lys Glu Glu Trp	Lys Ser Gln Glu Lys	Ser Pro Glu Lys
1595	1600	1605
Thr Ala Phe Lys Lys Lys	Asp Thr Ile Leu Ser	Leu Asn Ala Cys
1610	1615	1620
Glu Ser Asn His Ala Ile	Ala Ala Ile Asn Glu	Gly Gln Asn Lys
1625	1630	1635
Pro Glu Ile Glu Val Thr	Trp Ala Lys Gln Gly	Arg Thr Glu Arg
1640	1645	1650
Leu Cys Ser Gln Asn Pro	Pro Val Leu Lys Arg	His Gln Arg Glu
1655	1660	1665
Ile Thr Arg Thr Thr Leu	Gln Ser Asp Gln Glu	Glu Ile Asp Tyr
1670	1675	1680
Asp Asp Thr Ile Ser Val	Glu Met Lys Lys Glu	Asp Phe Asp Ile
1685	1690	1695
Tyr Asp Glu Asp Glu Asn	Gln Ser Pro Arg Ser	Phe Gln Lys Lys
1700	1705	1710
Thr Arg His Tyr Phe Ile	Ala Ala Val Glu Arg	Leu Trp Asp Tyr
1715	1720	1725
Gly Met Ser Ser Ser Pro	His Val Leu Arg Asn	Arg Ala Gln Ser
1730	1735	1740
Gly Ser Val Pro Gln Phe	Lys Lys Val Val Phe	Gln Glu Phe Thr
1745	1750	1755

Asp Gly 1760	Ser Phe Thr Gln	Pro 1765	Leu Tyr Arg Gly	Glu 1770	Leu Asn Glu
His Leu 1775	Gly Leu Leu Gly	Pro 1780	Tyr Ile Arg Ala	Glu 1785	Val Glu Asp
Asn Ile 1790	Met Val Thr Phe	Arg 1795	Asn Gln Ala Ser	Arg 1800	Pro Tyr Ser
Phe Tyr 1805	Ser Ser Leu Ile	Ser 1810	Tyr Glu Glu Asp	Gln 1815	Arg Gln Gly
Ala Glu 1820	Pro Arg Lys Asn	Phe 1825	Val Lys Pro Asn	Glu 1830	Thr Lys Thr
Tyr Phe 1835	Trp Lys Val Gln	His 1840	His Met Ala Pro	Thr 1845	Lys Asp Glu
Phe Asp 1850	Cys Lys Ala Trp	Ala 1855	Tyr Phe Ser Asp	Val 1860	Asp Leu Glu
Lys Asp 1865	Val His Ser Gly	Leu 1870	Ile Gly Pro Leu	Leu 1875	Val Cys His
Thr Asn 1880	Thr Leu Asn Pro	Ala 1885	His Gly Arg Gln	Val 1890	Thr Val Gln
Glu Phe 1895	Ala Leu Phe Phe	Thr 1900	Ile Phe Asp Glu	Thr 1905	Lys Ser Trp
Tyr Phe 1910	Thr Glu Asn Met	Glu 1915	Arg Asn Cys Arg	Ala 1920	Pro Cys Asn
Ile Gln 1925	Met Glu Asp Pro	Thr 1930	Phe Lys Glu Asn	Tyr 1935	Arg Phe His
Ala Ile 1940	Asn Gly Tyr Ile	Met 1945	Asp Thr Leu Pro	Gly 1950	Leu Val Met
Ala Gln 1955	Asp Gln Arg Ile	Arg 1960	Trp Tyr Leu Leu	Ser 1965	Met Gly Ser
Asn Glu 1970	Asn Ile His Ser	Ile 1975	His Phe Ser Gly	His 1980	Val Phe Thr
Val Arg 1985	Lys Lys Glu Glu	Tyr 1990	Lys Met Ala Leu	Tyr 1995	Asn Leu Tyr
Pro Gly 2000	Val Phe Glu Thr	Val 2005	Glu Met Leu Pro	Ser 2010	Lys Ala Gly
Ile Trp 2015	Arg Val Glu Cys	Leu 2020	Ile Gly Glu His	Leu 2025	His Ala Gly
Met Ser 2030	Thr Leu Phe Leu	Val 2035	Tyr Ser Asn Lys	Cys 2040	Gln Thr Pro
Leu Gly 2045	Met Ala Ser Gly	His 2050	Ile Arg Asp Phe	Gln 2055	Ile Thr Ala

Ser Gly	Gln Tyr Gly Gln	Trp	Ala Pro Lys Leu	Ala	Arg Leu His
2060		2065		2070	
Tyr Ser	Gly Ser Ile Asn	Ala	Trp Ser Thr Lys	Glu	Pro Phe Ser
2075		2080		2085	
Trp Ile	Lys Val Asp Leu	Leu	Ala Pro Met Ile	Ile	His Gly Ile
2090		2095		2100	
Lys Thr	Gln Gly Ala Arg	Gln	Lys Phe Ser Ser	Leu	Tyr Ile Ser
2105		2110		2115	
Gln Phe	Ile Ile Met Tyr	Ser	Leu Asp Gly Lys	Lys	Trp Gln Thr
2120		2125		2130	
Tyr Arg	Gly Asn Ser Thr	Gly	Thr Leu Met Val	Phe	Phe Gly Asn
2135		2140		2145	
Val Asp	Ser Ser Gly Ile	Lys	His Asn Ile Phe	Asn	Pro Pro Ile
2150		2155		2160	
Ile Ala	Arg Tyr Ile Arg	Leu	His Pro Thr His	Tyr	Ser Ile Arg
2165		2170		2175	
Ser Thr	Leu Arg Met Glu	Leu	Met Gly Cys Asp	Leu	Asn Ser Cys
2180		2185		2190	
Ser Met	Pro Leu Gly Met	Glu	Ser Lys Ala Ile	Ser	Asp Ala Gln
2195		2200		2205	
Ile Thr	Ala Ser Ser Tyr	Phe	Thr Asn Met Phe	Ala	Thr Trp Ser
2210		2215		2220	
Pro Ser	Lys Ala Arg Leu	His	Leu Gln Gly Arg	Ser	Asn Ala Trp
2225		2230		2235	
Arg Pro	Gln Val Asn Asn	Pro	Lys Glu Trp Leu	Gln	Val Asp Phe
2240		2245		2250	
Gln Lys	Thr Met Lys Val	Thr	Gly Val Thr Thr	Gln	Gly Val Lys
2255		2260		2265	
Ser Leu	Leu Thr Ser Met	Tyr	Val Lys Glu Phe	Leu	Ile Ser Ser
2270		2275		2280	
Ser Gln	Asp Gly His Gln	Trp	Thr Leu Phe Phe	Gln	Asn Gly Lys
2285		2290		2295	
Val Lys	Val Phe Gln Gly	Asn	Gln Asp Ser Phe	Thr	Pro Val Val
2300		2305		2310	
Asn Ser	Leu Asp Pro Pro	Leu	Leu Thr Arg Tyr	Leu	Arg Ile His
2315		2320		2325	
Pro Gln	Ser Trp Val His	Gln	Ile Ala Leu Arg	Met	Glu Val Leu
2330		2335		2340	
Gly Cys	Glu Ala Gln Asp	Leu	Tyr		
2345		2350			

&lt;210&gt; 31

&lt;211&gt; 1471

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 31

```

atggcgccccg tcgccgtctg ggccgcgctg gccgtcggac tggagctctg ggctgcggcg      60
cacgccttgc ccgcccaggt ggcatttaca ccctacgccc cggagccccg gagcacatgc      120
cggctcagag aatactatga ccagacagct cagatgtgct gcagcaaattg ctgcgggggc      180
caacatgcaa aagtcttctg taccaagacc tcggacaccg tgtgtgactc ctgtgaggac      240
agcacataca ccagctctg gaactgggtt cccgagtgtc tgagctgtgg ctcccgtgtg      300
agctctgacc aggtggaaac tcaagcctgc actcgggaac agaaccgcat ctgcacctgc      360
aggcccggtt ggtactgcgc gctgagcaag caggaggggt gccggtgtg cgcgcgctg      420
cgcaagtgcc gcccgggctt cggcgtggcc agaccaggaa ctgaaacatc agacgtggtg      480
tgcaagccct gtgccccggg gacgttctcc aacacgactt catccacgga tatttgagg      540
ccccaccaga tctgtaacgt ggtggccatc cctgggaatg caagcatgga tgcagtctgc      600
acgtccacgt cccccaccg gagtatggcc ccaggggcag tacacttacc ccagccagtg      660
tccacacgat cccaacacac gcagccaact ccagaaccca gcactgctcc aagcacctcc      720
ttcctgctcc caatgggccc cagcccccca gctgaaggga gcactggcga cttcgtctct      780
ccagttggac tgattgtggg tgtgacagcc ttgggtctac taataatagg agtgggtgaac      840
tgtgtcatca tgaccaggt gaaaaagaag cccttgtgcc tgacagagaga agccaagggtg      900
cctcacttgc ctgccgataa ggccccgggt acacagggcc ccgagcagca gcacctgctg      960
atcacagcgc cgagctccag cagcagctcc ctggagagct cggccagtgc gttggacaga      1020
agggcgcccc ctcggaacca gccacaggca ccaggcgtgg aggccagtgg ggccggggag      1080
ggccggggcca gcaccgggag ctcagattct tcccctggtg gccatgggac ccagggtcaat      1140
gtcacctgca tcgtgaacgt ctgtagcagc tctgaccaca gtcacagtgc ctctcccaa      1200
gccagctcca caatgggaga cacagattcc agcccctcgg agtccccgaa ggacgagcag      1260
gtcccccttct ccaaggagga atgtgccttt cggtcacagc tggagacgcc agagaccctg      1320
ctgggggagca ccgaagagaa gcccctgccc cttggagtgc ctgatgctgg gatgaagccc      1380
agttaaccag gccggtgtgg gctgtgtcgt agccaagggt ggctgagccc tggcaggatg      1440
accctgcgaa ggggccctgg tccttccagg c

```

&lt;210&gt; 32

&lt;211&gt; 461

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 32

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu  
 1 5 10 15  
 Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr  
 20 25 30  
 Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln  
 35 40 45  
 Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys  
 50 55 60  
 Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp  
 65 70 75 80  
 Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys  
 85 90 95  
 Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg  
 100 105 110  
 Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu  
 115 120 125  
 Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg  
 130 135 140  
 Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val  
 145 150 155 160  
 Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr  
 165 170 175  
 Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly  
 180 185 190  
 Asn Ala Ser Met Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser  
 195 200 205  
 Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser  
 210 215 220  
 Gln His Thr Gln Pro Thr Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser  
 225 230 235 240  
 Phe Leu Leu Pro Met Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly  
 245 250 255  
 Asp Phe Ala Leu Pro Val Gly Leu Ile Val Gly Val Thr Ala Leu Gly  
 260 265 270  
 Leu Leu Ile Ile Gly Val Val Asn Cys Val Ile Met Thr Gln Val Lys  
 275 280 285  
 Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val Pro His Leu Pro  
 290 295 300  
 Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu Gln Gln His Leu Leu  
 305 310 315 320  
 Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser

	325		330		335
Ala Leu Asp Arg Arg Ala Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly					
	340		345		350
Val Glu Ala Ser Gly Ala Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser					
	355		360		365
Asp Ser Ser Pro Gly Gly His Gly Thr Gln Val Asn Val Thr Cys Ile					
	370		375		380
Val Asn Val Cys Ser Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln					
	385		390		400
Ala Ser Ser Thr Met Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro					
	405		410		415
Lys Asp Glu Gln Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser					
	420		425		430
Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro					
	435		440		445
Leu Pro Leu Gly Val Pro Asp Ala Gly Met Lys Pro Ser					
	450		455		460

<210> 33  
 <211> 1475  
 <212> DNA  
 <213> Homo sapiens

<400> 33  
 tccacctgtc cccgcagcgc cggctcgcgc cctcctgccg cagccaccga gccgccgtct 60  
 agcgccccga cctcgccacc atgagagccc tgctggcgcg cctgcttctc tgcgtcctgg 120  
 tcgtgagcga ctccaaaggc agcaatgaac ttcatcaagt tccatcgaac tgtgactgtc 180  
 taaatggagg aacatgtgtg tccaacaagt acttctccaa cattcactgg tgcaactgcc 240  
 caaagaaatt cggagggcag cactgtgaaa tagataagtc aaaaacctgc tatgagggga 300  
 atggtcactt ttaccgagga aaggccagca ctgacaccat gggccggccc tgccctgccct 360  
 ggaactctgc cactgtcctt cagcaaactg accatgccc aagatctgat gctcttcagc 420  
 tgggcctggg gaaacataat tactgcagga acccagacaa ccggaggcga ccctgggtgct 480  
 atgtgcaggt gggcctaaag ccgcttgtcc aagagtgcac ggtgcatgac tgcgcagatg 540  
 gaaaaaagcc ctccctctct ccagaagaat taaaatttca gtgtggccaa aagactctga 600  
 ggccccgctt taagattatt gggggagaat tcaccaccat cgagaaccag ccctgggttg 660  
 cggccatcta caggaggcac cgggggggct ctgtcaccta cgtgtgtgga ggcagcctca 720  
 tcagcccttg ctgggtgatc agcgccacac actgcttcat tgattaccca aagaaggagg 780  
 actacatcgt ctacctgggt cgctcaaggc ttaactccaa cacgcaaggg gagatgaagt 840  
 ttgaggtgga aaacctcctc ctacacaagg actacagcgc tgacacgctt gctcaccaca 900



acgacattgc cttgctgaag atccgttcca aggagggcag gtgtgcgag ccatcccgga 960  
 ctatacagac catctgcctg ccctcgatgt ataacgatcc ccagtttggc acaagctgtg 1020  
 agatcactgg ctttggaataa gagaattcta ccgactatct ctatccggag cagctgaaga 1080  
 tgactgttgt gaagctgatt tcccaccggg agtgtcagca gcccactac tacggctctg 1140  
 aagtcaccac caaaatgctg tgtgctgctg acccacagtg gaaaacagat tcctgccagg 1200  
 gagactcagg gggacccttc gtctgttccc tccaaggccg catgactttg actggaattg 1260  
 tgagctgggg ccgtggatgt gccctgaagg acaagccagg cgtctacacg agagtctcac 1320  
 acttcttacc ctggatccgc agtcacacca aggaagagaa tggcctggcc ctctgagggt 1380  
 ccccgaggag gaaacgggca ccaccgctt tcttgctggg tgtcattttt gcagtagagt 1440  
 catctccatc agctgtaaga agagactggg aagat 1475

<210> 34  
 <211> 431  
 <212> PRT  
 <213> Homo sapiens

<400> 34  
 Met Arg Ala Leu Leu Ala Arg Leu Leu Leu Cys Val Leu Val Val Ser  
 1 5 10 15  
 Asp Ser Lys Gly Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp  
 20 25 30  
 Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile  
 35 40 45  
 His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile  
 50 55 60  
 Asp Lys Ser Lys Thr Cys Tyr Glu Gly Asn Gly His Phe Tyr Arg Gly  
 65 70 75 80  
 Lys Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro Trp Asn Ser  
 85 90 95  
 Ala Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp Ala Leu  
 100 105 110  
 Gln Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Asn Arg  
 115 120 125  
 Arg Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu Val Gln  
 130 135 140  
 Glu Cys Met Val His Asp Cys Ala Asp Gly Lys Lys Pro Ser Ser Pro  
 145 150 155 160  
 Pro Glu Glu Leu Lys Phe Gln Cys Gly Gln Lys Thr Leu Arg Pro Arg  
 165 170 175  
 Phe Lys Ile Ile Gly Gly Glu Phe Thr Thr Ile Glu Asn Gln Pro Trp

```

<400> 35
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala
20 25 30
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

```

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro  
 85 90 95  
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
 100 105

<210> 36  
 <211> 120  
 <212> PRT  
 <213> Mus musculus

<400> 36  
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly  
 1 5 10 15  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr  
 20 25 30  
 Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
 35 40 45  
 Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val  
 50 55 60  
 Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr  
 65 70 75 80  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
 Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln  
 100 105 110  
 Gly Thr Leu Val Thr Val Ser Ser  
 115 120

<210> 37  
 <211> 120  
 <212> PRT  
 <213> Mus musculus

<400> 37  
 Gln Val Thr Leu Arg Glu Ser Gly Pro Ala Leu Val Lys Pro Thr Gln  
 1 5 10 15  
 Thr Leu Thr Leu Thr Cys Thr Phe Ser Gly Phe Ser Leu Ser Thr Ser  
 20 25 30  
 Gly Met Ser Val Gly Trp Ile Arg Gln Pro Ser Gly Lys Ala Leu Glu  
 35 40 45  
 Trp Leu Ala Asp Ile Trp Trp Asp Asp Lys Lys Asp Tyr Asn Pro Ser  
 50 55 60

Leu Lys Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val  
65 70 75 80

Val Leu Lys Val Thr Asn Met Asp Pro Ala Asp Thr Ala Thr Tyr Tyr  
85 90 95

Cys Ala Arg Ser Met Ile Thr Asn Trp Tyr Phe Asp Val Trp Gly Ala  
100 105 110

Gly Thr Thr Val Thr Val Ser Ser  
115 120

<210> 38

<211> 106

<212> PRT

<213> Mus musculus

<400> 38

Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Val Gly  
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Lys Cys Gln Leu Ser Val Gly Tyr Met  
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Trp Ile Tyr  
35 40 45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser  
50 55 60

Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Asp  
65 70 75 80

Asp Phe Ala Thr Tyr Tyr Cys Phe Gln Gly Ser Gly Tyr Pro Phe Thr  
85 90 95

Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys  
100 105

<210> 39

<211> 1039

<212> DNA

<213> Homo sapiens

<400> 39

tcctgcacag gcagtgcctt gaagtgcttc ttcagagacc tttcttcata gactactttt 60  
ttttcttttaa gcagcaaaag gagaaaattg tcatcaaagg atattccaga ttcttgaçag 120  
cattctcgtc atctctgagg acatcaccat catctcagga tgaggggcat gaagctgctg 180  
ggggcgctgc tggcaactggc ggccctactg cagggggccg tgtccctgaa gatcgagcc 240  
ttcaacatcc agacatttgg ggagaccaag atgtccaatg ccaccctcgt cagctacatt 300  
gtgcagatcc tgacccgcta tgacatcgcc ctggtccagg aggtcagaga cagccacctg 360  
actgccgtgg ggaagctgct ggacaacctc aatcaggatg caccagacac ctatcactac 420  
gtggtcagtg agccactggg acggaacagc tataaggagc gctacctgtt cgtgtacagg 480

cctgaccagg tgtctgcggt ggacagctac tactacgatg atggctgcga gccctgcggg 540  
 aacgacacct tcaaccgaga gccagccatt gtcaggttct tctcccgggt cacagaggtc, 600  
 agggagtttg ccattgttcc cctgcatgcg gccccggggg acgcagtagc cgagatcgac 660  
 gctctctatg acgtctacct ggatgtccaa gagaaatggg gcttggagga cgtcatgttg 720  
 atgggagcact tcaatgcggg ctgcagctat gtgagaccct cccagtgggtc atccatccgc 780  
 ctgtggacaa gccccacctt ccagtggctg atccccgaca gcgctgacac cacagctaca 840  
 cccacgcact gtgcctatga caggatcgtg gttgcaggga tgctgctccg aggcgccgtt 900  
 gttcccgaact cggtcttcc ctttaacttc caggctgcct atggcctgag tgaccaactg 960  
 gcccgaagcca tcagtgaacca ctatccagtg gaggtgatgc tgaagtgagc agcccctccc 1020  
 cacaccagtt gaactgcag 1039

<210> 40  
 <211> 282  
 <212> PRT  
 <213> Homo sapiens

<400> 40  
 Met Arg Gly Met Lys Leu Leu Gly Ala Leu Leu Ala Leu Ala Ala Leu  
 1 5 10 15  
 Leu Gln Gly Ala Val Ser Leu Lys Ile Ala Ala Phe Asn Ile Gln Thr  
 20 25 30  
 Phe Gly Glu Thr Lys Met Ser Asn Ala Thr Leu Val Ser Tyr Ile Val  
 35 40 45  
 Gln Ile Leu Ser Arg Tyr Asp Ile Ala Leu Val Gln Glu Val Arg Asp  
 50 55 60  
 Ser His Leu Thr Ala Val Gly Lys Leu Leu Asp Asn Leu Asn Gln Asp  
 65 70 75 80  
 Ala Pro Asp Thr Tyr His Tyr Val Val Ser Glu Pro Leu Gly Arg Asn  
 85 90 95  
 Ser Tyr Lys Glu Arg Tyr Leu Phe Val Tyr Arg Pro Asp Gln Val Ser  
 100 105 110  
 Ala Val Asp Ser Tyr Tyr Tyr Asp Asp Gly Cys Glu Pro Cys Gly Asn  
 115 120 125  
 Asp Thr Phe Asn Arg Glu Pro Ala Ile Val Arg Phe Phe Ser Arg Phe  
 130 135 140  
 Thr Glu Val Arg Glu Phe Ala Ile Val Pro Leu His Ala Ala Pro Gly  
 145 150 155 160  
 Asp Ala Val Ala Glu Ile Asp Ala Leu Tyr Asp Val Tyr Leu Asp Val  
 165 170 175  
 Gln Glu Lys Trp Gly Leu Glu Asp Val Met Leu Met Gly Asp Phe Asn  
 180 185 190

Ala Gly Cys Ser Tyr Val Arg Pro Ser Gln Trp Ser Ser Ile Arg Leu  
 195 200 205

Trp Thr Ser Pro Thr Phe Gln Trp Leu Ile Pro Asp Ser Ala Asp Thr  
 210 215 220

Thr Ala Thr Pro Thr His Cys Ala Tyr Asp Arg Ile Val Val Ala Gly  
 225 230 235 240

Met Leu Leu Arg Gly Ala Val Val Pro Asp Ser Ala Leu Pro Phe Asn  
 245 250 255

Phe Gln Ala Ala Tyr Gly Leu Ser Asp Gln Leu Ala Gln Ala Ile Ser  
 260 265 270

Asp His Tyr Pro Val Glu Val Met Leu Lys  
 275 280

<210> 41  
 <211> 678  
 <212> DNA  
 <213> Mus musculus

<400> 41  
 gacatcttgc tgactcagtc tccagccatc ctgtctgtga gtccaggaga aagagtcagt 60  
 ttctcctgca gggccagtc gttcgttggc tcaagcatcc actggtatca gcaaagaaca 120  
 aatggttctc caaggcttct cataaagtat gcttctgagt ctatgtctgg gatcccttcc 180  
 aggttttagtg gcagtggatc agggacagat tttactctta gcatcaacac tgtggagtct 240  
 gaagatattg cagattatta ctgtcaacaa agtcatagct ggccattcac gttcggctcg 300  
 gggacaaatt tggaagtaaa agaagtgaag cttgaggagt ctggaggagg cttggtgcaa 360  
 cctggaggat ccatgaaact ctctgtgtt gcctctggat tcattttcag taaccactgg 420  
 atgaactggg tccgccagtc tccagagaag gggcttgagt gggttgctga aattagatca 480  
 aaatctatta attctgcaac acattatgcg gagtctgtga aaggagggtt caccatctca 540  
 agagatgatt ccaaaagtgc tgtctacctg caaatgaccg acttaagaac tgaagacact 600  
 ggcgtttatt actgttccag gaattactac ggtagtacct acgactactg gggccaaggc 660  
 accactctca cagtctcc 678

<210> 42  
 <211> 226  
 <212> PRT  
 <213> Mus musculus

<400> 42  
 Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser Val Ser Pro Gly  
 1 5 10 15  
 Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Phe Val Gly Ser Ser  
 20 25 30

<400>	43						
gctgcatcag	aagaggccat	caagcacatc	actgtccttc	tgccatggcc	ctgtgggatgc		60
gcctcctgcc	cctgctggcg	ctgctggccc	tctggggacc	tgaccagcc	gcagcctttg		120
tgaaccaaca	cctgtgcggc	tcacacctg	tggaagctct	ctacctagt	tgcggggaac		180
gaggcttctt	ctacacaccc	aagaccgcc	gggaggcaga	ggacctgcag	gtggggcagg		240
tggagctggg	cgggggccct	ggtgcaggca	gcctgcagcc	cttggccctg	gaggggtccc		300
tgcagaagcg	tggcattgtg	gaacaatgct	gtaccagcat	ctgctccctc	taccagctgg		360
agaactactg	caactagacg	cagcccgcag	gcagccccc	accgcgcgc	tcctgcaccg		420
agagagatgg	aataaagccc	ttgaaccagc					450

<210> 44  
 <211> 110  
 <212> PRT  
 <213> Homo sapiens

<400> 44  
 Met Ala Leu Trp Met Arg Leu Leu Pro Leu Leu Ala Leu Leu Ala Leu  
 1 5 10 15  
 Trp Gly Pro Asp Pro Ala Ala Ala Phe Val Asn Gln His Leu Cys Gly  
 20 25 30  
 Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe  
 35 40 45  
 Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly  
 50 55 60  
 Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu  
 65 70 75 80  
 Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys  
 85 90 95  
 Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn  
 100 105 110

<210> 45  
 <211> 1203  
 <212> DNA  
 <213> Hepatitis B virus

<400> 45  
 atgggagggtt ggtcttccaa acctcgacaa ggcattgggga cgaatctttc tgttcccaat 60  
 cctctgggat tctttccoga tcaccagttg gaccctgcgt tcggagccaa ctcaaacaat 120  
 ccagattggg acttcaaccc caacaaggat cactggccag aggcaatcaa ggtaggagcg 180  
 ggagacttgc ggccagggtt caccacacca caaggcggtc ttttggggtg gagccctcag 240  
 gctcagggca tattgacaac agtgccagca ggcctcctc ctgtttccac caatcggcag 300  
 tcaggaagac agcctactcc catctctcca cctctaagag acagtcattc tcaggccatg 360  
 cagtggaaact ccacaacatt ccaccaagct ctgctagatc ccagagttag ggcctatat 420  
 tttctgctg gtggtccag ttccggaaca gtaaaccctg ttccgactac tgtctcacc 480  
 atatcgtcaa tcttctcgag gactggggac cctgcaccga acatggagag cacaacatca 540  
 ggattcctag gaccctgct cgtgttacag ggggggtttt tcttgttgac aagaatcctc 600  
 acaataccac agagtctaga ctctgtgttg acttctctca attttctagg gggagcacc 660  
 acgtgtcctg gccaaaattc gcagtcccca acctccaatc actcacciaac ctctgtcct 720  
 ccaatttctc ctggttatcg ctggatgtgt ctggggcggt ttatcatatt cctcttcac 780  
 ctgctgctat gcctcatctt cttgttggtt cttctggact accaagggtat gttgcccggt 840



tgtcctctac ttccaggaac atcaactacc agcacgggac catgcaagac ctgcacgatt 900  
 cctgctcaag gaacctctat gtttccctct tgttgctgta caaaaccttc ggacggaaac 960  
 tgcacttgta ttcccatccc atcatcctgg gctttcgcaa gattcctatg ggagtgggcc 1020  
 tcagtccgtt tctcctggct cagtttacta gtgccatttg ttcagtgggt cgcagggctt 1080  
 tcccccaactg ttgggctttc agttatatgg atgatgtggt attggggggcc aagtctgtac 1140  
 aacatcttga gtcccttttt acctctatta ccaattttct tttgtctttg ggtatacatt 1200  
 tga 1203

<210> 46  
 <211> 400  
 <212> PRT  
 <213> Hepatitis B virus

<400> 46  
 Met Gly Gly Trp Ser Ser Lys Pro Arg Gln Gly Met Gly Thr Asn Leu  
 1 5 10 15  
 Ser Val Pro Asn Pro Leu Gly Phe Phe Pro Asp His Gln Leu Asp Pro  
 20 25 30  
 Ala Phe Gly Ala Asn Ser Asn Asn Pro Asp Trp Asp Phe Asn Pro Asn  
 35 40 45  
 Lys Asp His Trp Pro Glu Ala Ile Lys Val Gly Ala Gly Asp Phe Gly  
 50 55 60  
 Pro Gly Phe Thr Pro Pro His Gly Gly Leu Leu Gly Trp Ser Pro Gln  
 65 70 75 80  
 Ala Gln Gly Ile Leu Thr Thr Val Pro Ala Ala Pro Pro Pro Val Ser  
 85 90 95  
 Thr Asn Arg Gln Ser Gly Arg Gln Pro Thr Pro Ile Ser Pro Pro Leu  
 100 105 110  
 Arg Asp Ser His Pro Gln Ala Met Gln Trp Asn Ser Thr Thr Phe His  
 115 120 125  
 Gln Ala Leu Leu Asp Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly  
 130 135 140  
 Gly Ser Ser Ser Gly Thr Val Asn Pro Val Pro Thr Thr Val Ser Pro  
 145 150 155 160  
 Ile Ser Ser Ile Phe Ser Arg Thr Gly Asp Pro Ala Pro Asn Met Glu  
 165 170 175  
 Ser Thr Thr Ser Gly Phe Leu Gly Pro Leu Leu Val Leu Gln Ala Gly  
 180 185 190  
 Phe Phe Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp Ser  
 195 200 205  
 Trp Trp Thr Ser Leu Asn Phe Leu Gly Gly Ala Pro Thr Cys Pro Gly  
 210 215 220

Gln Asn Ser Gln Ser Pro Thr Ser Asn His Ser Pro Thr Ser Cys Pro  
 225 230 235 240  
 Pro Ile Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe Ile Ile  
 245 250 255  
 Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val Leu Leu  
 260 265 270  
 Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Leu Pro Gly Thr Ser  
 275 280 285  
 Thr Thr Ser Thr Gly Pro Cys Lys Thr Cys Thr Ile Pro Ala Gln Gly  
 290 295 300  
 Thr Ser Met Phe Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly Asn  
 305 310 315 320  
 Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Ala Arg Phe Leu  
 325 330 335  
 Trp Glu Trp Ala Ser Val Arg Phe Ser Trp Leu Ser Leu Leu Val Pro  
 340 345 350  
 Phe Val Gln Trp Phe Ala Gly Leu Ser Pro Thr Val Trp Leu Ser Val  
 355 360 365  
 Ile Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Asn Ile Leu Ser  
 370 375 380  
 Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val Tyr Ile  
 385 390 395 400

&lt;210&gt; 47

&lt;211&gt; 799

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 47

cgaaccactc agggctcctgt ggacagctca cctagctgca atggctacag gctcccggac 60  
 gtccctgctc ctggcttttg gctgctctg cctgccttg cttcaagagg gcagtgcctt 120  
 cccaaccatt cccttatcca ggcttttga caacgctatg ctccgcgcc atcgtctgca 180  
 ccagctggcc ttgacacct accaggagtt tgaagaagcc tatatcccaa aggaacagaa 240  
 gtattcattc ctgcagaacc ccagacctc cctctgttgc tcagagtcta ttccgacacc 300  
 ctccaacagg gaggaaacac aacagaaatc caacctagag ctgctccgca tctccctgct 360  
 gctcatccag tcgtggctgg agcccgctga gttcctcagg agtgtcttcg ccaacagcct 420  
 ggtgtacggc gcctctgaca gcaacgtcta tgacctccta aaggacctag aggaaggcat 480  
 ccaaacgctg atggggaggc tggaagatgg cagcccccg actgggcaga tcttcaagca 540  
 gacctacagc aagttcgaca caaactcaca caacgatgac gcactactca agaactacgg 600  
 gctgctctac tgcttcagga aggacatgga caaggctcgag acattcctgc gcacgtgca 660

gtgccgctct gtggaggga gctgtggctt ctagctgcc cgggtggcatc cctgtgaccc 720  
 ctccccagtg cctctcctgg ccctggaagt tgccactcca gtgcccacca gccttgctct 780  
 aataaaatta agttgcatc 799

<210> 48  
 <211> 217  
 <212> PRT  
 <213> Homo sapiens

<400> 48  
 Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu  
 1 5 10 15  
 Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu  
 20 25 30  
 Ser Arg Pro Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln  
 35 40 45  
 Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys  
 50 55 60  
 Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe  
 65 70 75 80  
 Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys  
 85 90 95  
 Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp  
 100 105 110  
 Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val  
 115 120 125  
 Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu  
 130 135 140  
 Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg  
 145 150 155 160  
 Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser  
 165 170 175  
 His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe  
 180 185 190  
 Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys  
 195 200 205  
 Arg Ser Val Glu Gly Ser Cys Gly Phe  
 210 215

<210> 49  
 <211> 963  
 <212> DNA  
 <213> Homo sapiens

<400> 49

atggagacag acacactcct gttatgggtg ctgctgctct gggttccagg ttccactggg 60  
 gacgtcaggc gagggccccg gagcctgcgg ggcaggagac cgccagcccc cacgccctgc 120  
 gtcccggccg agtgcttcga cctgctggtc cgccactgcg tggcctgcgg gtcctctgcg 180  
 acgccgcggc cgaaccggc cggggccagc agccctgcgc ccaggacggc gctgcagccg 240  
 caggagtcgg tgggcgcggg ggccggcgag ggcggcgtcg acaaaactca cacatgccca 300  
 ccgtgcccag cacctgaact cctgggggga cgtcagtcct tcctcttccc cccaaaaccc 360  
 aaggacaccc tcatgatctc ccggaccctt gaggtcacat gcgtgggtgt ggacgtgagc 420  
 cacgaagacc ctgaggtcaa gttcaactgg tacgtggacg gcgtggaggt gcataatgcc 480  
 aagacaaagc cgccgggagga gcagtacaac agcacgtacc gtgtggtcag cgtcctcacc 540  
 gtctctgcacc aggactggct gaatggcaag gagtacaagt gcaaggctct caacaaagcc 600  
 ctcccagccc ccatcgagaa aaccatctcc aaagcctaaag ggcagccccg agaaccacag 660  
 gtgtacaccc tgccccatc ccgggatgag ctgaccaaga accaggtcag cctgacctgc 720  
 ctgggtcaaag gcttctatcc cagcgacatc gccgtggagt gggagagcaa tgggcagccg 780  
 gagaacaact acaagaccac gcctcccggtg ttggactccg acggctcctt ctctctctac 840  
 agcaagctca ccgtggacaa gagcagggtg cagcagggga acgtcttctc atgctccgtg 900  
 atgcatgagg ctctgcacaa ccactacacg cagaagagcc tctccctgtc tcccgggaaa 960  
 tga 963

<210> 50  
 <211> 320  
 <212> PRT  
 <213> Homo sapiens

<400> 50  
 Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro  
 1 5 10 15  
 Gly Ser Thr Gly Asp Val Arg Arg Gly Pro Arg Ser Leu Arg Gly Arg  
 20 25 30  
 Asp Ala Pro Ala Pro Thr Pro Cys Val Pro Ala Glu Cys Phe Asp Leu  
 35 40 45  
 Leu Val Arg His Cys Val Ala Cys Gly Leu Leu Arg Thr Pro Arg Pro  
 50 55 60  
 Lys Pro Ala Gly Ala Ser Ser Pro Ala Pro Arg Thr Ala Leu Gln Pro  
 65 70 75 80  
 Gln Glu Ser Val Gly Ala Gly Ala Gly Glu Ala Ala Val Asp Lys Thr  
 85 90 95  
 His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser  
 100 105 110

Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg  
 115 120 125  
 Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro  
 130 135 140  
 Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala  
 145 150 155 160  
 Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val  
 165 170 175  
 Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr  
 180 185 190  
 Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr  
 195 200 205  
 Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu  
 210 215 220  
 Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys  
 225 230 235 240  
 Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser  
 245 250 255  
 Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp  
 260 265 270  
 Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser  
 275 280 285  
 Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala  
 290 295 300  
 Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys  
 305 310 315 320

<210> 51  
 <211> 107  
 <212> PRT  
 <213> Homo sapiens

<400> 51  
 Asp Ile Gln Met Thr Gln Thr Pro Ser Thr Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr  
 20 25 30  
 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
 35 40 45  
 Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 Asp Asp Phe Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro Trp

85 90 95  
 Thr Phe Gly Gln Gly Thr Lys Val Glu Val Lys  
 100 105  
 <210> 52  
 <211> 107  
 <212> PRT  
 <213> Mus musculus  
 <400> 52  
 Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr  
 20 25 30  
 Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Ile Val Lys Leu Leu Ile  
 35 40 45  
 Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Gln  
 65 70 75 80  
 Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro Trp  
 85 90 95  
 Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys  
 100 105  
 <210> 53  
 <211> 119  
 <212> PRT  
 <213> Homo sapiens  
 <400> 53  
 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser  
 1 5 10 15  
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Tyr  
 20 25 30  
 Leu Ile Glu Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile  
 35 40 45  
 Gly Val Ile Tyr Pro Gly Ser Gly Gly Thr Asn Tyr Asn Glu Lys Phe  
 50 55 60  
 Lys Gly Arg Val Thr Leu Thr Val Asp Glu Ser Thr Asn Thr Ala Tyr  
 65 70 75 80  
 Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys  
 85 90 95  
 Ala Arg Arg Asp Gly Asn Tyr Gly Trp Phe Ala Tyr Trp Gly Gln Gly  
 100 105 110  
 Thr Leu Val Thr Val Ser Ser  
 115

<210> 54  
 <211> 119  
 <212> PRT  
 <213> Mus musculus

<400> 54  
 Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Gly Pro Gly Thr  
 1 5 10 15  
 Ser Val Arg Val Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Tyr  
 20 25 30  
 Leu Ile Glu Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile  
 35 40 45  
 Gly Val Ile Tyr Pro Gly Ser Gly Gly Thr Asn Tyr Asn Glu Lys Phe  
 50 55 60  
 Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Thr Thr Ala Tyr  
 65 70 75 80  
 Met Gln Leu Ser Ser Leu Thr Ser Asp Asp Ser Ala Val Tyr Phe Cys  
 85 90 95  
 Ala Arg Arg Asp Gly Asn Tyr Gly Trp Phe Ala Tyr Trp Gly Arg Gly  
 100 105 110  
 Thr Leu Val Thr Val Ser Ala  
 115

<210> 55  
 <211> 214  
 <212> PRT  
 <213> Homo sapiens

<400> 55  
 Asp Ile Gln Met Thr Gln Thr Pro Ser Thr Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr  
 20 25 30  
 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
 35 40 45  
 Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 Asp Asp Phe Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro Trp  
 85 90 95  
 Thr Phe Gly Gln Gly Thr Lys Val Glu Val Lys Arg Thr Val Ala Ala  
 100 105 110  
 Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
 115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
195 200 205

Phe Asn Arg Gly Glu Cys  
210

<210> 56

<211> 448

<212> PRT

<213> Homo sapiens

<400> 56

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser  
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Tyr  
20 25 30

Leu Ile Glu Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile  
35 40 45

Gly Val Ile Tyr Pro Gly Ser Gly Gly Thr Asn Tyr Asn Glu Lys Phe  
50 55 60

Lys Gly Arg Val Thr Leu Thr Val Asp Glu Ser Thr Asn Thr Ala Tyr  
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys  
85 90 95

Ala Arg Arg Asp Gly Asn Tyr Gly Trp Phe Ala Tyr Trp Gly Gln Gly  
100 105 110

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
115 120 125

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
130 135 140

Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp  
145 150 155 160

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu  
165 170 175

Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser  
180 185 190

Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro



195	200	205
Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys 210 215 220		
Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro 225 230 235 240		
Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser 245 250 255		
Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp 260 265 270		
Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn 275 280 285		
Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val 290 295 300		
Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu 305 310 315 320		
Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys 325 330 335		
Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr 340 345 350		
Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr 355 360 365		
Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu 370 375 380		
Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu 385 390 395 400		
Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys 405 410 415		
Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu 420 425 430		
Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly 435 440 445		

&lt;210&gt; 57

&lt;211&gt; 8540

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 57

gacgtcgcgg cgcctctagg cctccaaaaa agcctcctca ctacttctgg aatagctcag 60

aggccgaggg ggcctcggcc tctgcataaa taaaaaaaat tagtcagcca tgcattggggc 120

ggagaatggg cggaactggg cggagttagg ggcgggatgg gcggagttag gggcggggact 180

atggttgctg actaattgag atgcatgctt tgcatacttc tgcctgctgg ggagcctggg 240

gactttccac acctggttgc tgactaattg agatgcatgc tttgcatact tctgcctgct 300  
 ggggagcctg gggactttcc acaccctaac tgacacacat tccacagaat taattcccct 360  
 agttattaat agtaatcaat tacgggggtca ttagttcata gcccatatat ggagttccgc 420  
 gttacataac ttacggtaaa tggcccgctt ggctgaccgc ccaacgaccc ccgcccattg 480  
 acgtcaataa tgacgtatgt tcccatagta acgccaatag ggactttcca ttgacgtcaa 540  
 tgggtggact atttacggta aactgcccac ttggcagtac atcaagtgtg tcatatgccca 600  
 agtacgcccc ctattgacgt caatgacggg aaatggcccg cctggcatta tgcccagtac 660  
 atgaccttat gggactttcc tacttggcag tacatctacg tattagtcac cgctattacc 720  
 atggtgatgc ggttttggca gtacatcaat gggcgtggat agcggtttga ctcacgggga 780  
 tttccaagtc tccaccccat tgacgtcaat gggagtttgt tttggcacca aaatcaacgg 840  
 gactttccaa aatgtcgtaa caactccgcc ccattgacgc aaatgggagg taggcgtgta 900  
 cggtgggagg tctatataag cagagctggg tacgtgaacc gtcagatcgc ctggagacgc 960  
 catcacagat ctctcaccat gaggggtccc gctcagctcc tgggggtcct gctgctctgg 1020  
 ctcccagggt cagcatgtga tgggtaccaag gtggaaatca aacgtacggg ggctgcacca 1080  
 tctgtcttca tcttcccgcc atctgatgag cagttgaaat ctggaactgc ctctgttgtg 1140  
 tgctgtctga ataacttcta tcccagagag gccaaagtac agtggaaggt ggataacgcc 1200  
 ctccaatcgg gtaactccca ggagagtgtc acagagcagg acagcaagga cagcacctac 1260  
 agcctcagca gcaccctgac gctgagcaaa gcagaetacg agaaacacaa agtctacgcc 1320  
 tgcgaagtca cccatcaggg cctgagctcg cccgtcacia agagcttcaa caggggagag 1380  
 tgttgaattc agatccgtta acggttacca actacctaga ctggattcgt gacaacatgc 1440  
 ggccgtgata tctacgtatg atcagcctcg actgtgcctt ctagttagcca gccatctgtt 1500  
 gtttggccct ccccggtgcc ttccttgacc ctggaagggt ccaactccac tgtcctttcc 1560  
 taataaaatg aggaaattgc atcgcatgtg ctgagtaggt gtcattctat tctggggggg 1620  
 ggggtggggc aggacagcaa gggggaggat tgggaagaca atagcaggca tgctggggat 1680  
 gcggtgggct ctatggaacc agctggggct cgacagctat gccaagtacg cccctattg 1740  
 acgtcaatga cggtaaatgg cccgcctggc attatgcccc gtacatgacc ttatgggact 1800  
 ttctacttg gcagtacatc tacgtattag tcatcgctat taccatgggt atgcggtttt 1860  
 ggcagtacat caatgggctg ggatagcggg ttgactcacg gggatttcca agtctccacc 1920  
 ccattgacgt caatgggagt ttgttttggc accaaaatca acgggacttt ccaaaatgtc 1980  
 gtaacaactc cggcccattg acgcaaatgg gcggtaggcg tgtacgggtg gaggtotata 2040  
 taagcagagc tgggtacgtc ctcacattca gtgatdagca ctgaacacag acccgctcagc 2100

atgggttgga gcctcatctt gctcttcctt gtcgctgttg ctacgcgtgt cgctagcacc 2160  
 aaggggcccat cggtcttccc cctggcacc cctccaaga gcacctctgg gggcacagcg 2220  
 gccctgggct gcctgggtcaa ggactacttc cccgaaccgg tgacggtgtc gtggaactca 2280  
 ggcgcctga ccagcggcgt gcacaccttc ccggtgtcc tacagtcctc aggactctac 2340  
 tccctcagca gcgtggtgac cgtgccctcc agcagcttg gcaccagac ctacatctgc 2400  
 aacgtgaatc acaagccag caacaccaag gtggacaaga aagcagagcc caaatcttgt 2460  
 gacaaaactc acacatgccc accgtgccc gcacctgaac tcctgggggg accgtcagtc 2520  
 ttctcttccc cccaaaacc caaggacacc ctcatgatct cccggacccc tgaggtcaca 2580  
 tgcgtggtgg tggacgtgag ccacgaagac cctgaggtca agttcaactg gtacgtggac 2640  
 ggcgtggagg tgcataatgc caagacaaag ccgcgggagg agcagtacaa cagcacgtac 2700  
 cgtgtggtca gcgtcctcac cgtcctgcac caggactggc tgaatggcaa ggactacaag 2760  
 tgcaaggtct ccaacaaagc cctcccagcc cccatcgaga aaaccatctc caaagccaaa 2820  
 gggcagcccc gagaaccaca ggtgtacacc ctgccccat cccgggatga gctgaccagg 2880  
 aaccagggtca gcctgacctg cctgggtcaa ggcttctatc ccagcgacat cgccgtggag 2940  
 tgggagagca atgggcagcc ggagaacaac tacaagacca cgctcccgt gctggactcc 3000  
 gacggctcct tcttctcta cagcaagctc accgtggaca agagcagggtg gcagcagggg 3060  
 aacgtcttct catgctcgt gatgcatgag gctctgcaca accactacac gcagaagagc 3120  
 ctctccctgt ctccgggtaa atgaggatcc gttaacggtt accaactacc tagactggat 3180  
 tcgtgacaac atgcggccgt gatattctacg tatgatcagc ctgactgtg ccttctagtt 3240  
 gccagccatc tgttgtttgc cctcccccg tgcttctctt gacctggaa ggtgccactc 3300  
 ccactgtcct ttctaataa aatgaggaaa ttgcatcgca ttgtctgagt aggtgtcatt 3360  
 ctattctggg ggggtgggtg gggcaggaca gcaaggggga ggattgggaa gacaatagca 3420  
 ggcatgctgg ggatgcggtg ggctctatgg aaccagctgg ggctcgacag cgctggatct 3480  
 cccgatcccc agctttgctt ctcaatttct tatttgcata atgagaaaaa aaggaaaatt 3540  
 aattttaaca ccaattcagt agttgattga gcaaagcgt tgccaaaag gatgctttag 3600  
 agacagtgtt ctctgcacag ataaggacaa acattattca gagggagtac ccagagctga 3660  
 gactcctaag ccagtgagtg gcacagcatt ctagggagaa atatgcttgt catcaccgaa 3720  
 gcctgattcc gtagagccac accttggtaa gggccaatct gctcacacag gatagagagg 3780  
 gcaggagcca gggcagagca tataaggtga ggtaggatca gttgctctc acatttgctt 3840  
 ctgacatagt tgtgttgga gcttgatag cttggacagc tcagggtgc gatttcgcgc 3900

caaacttgac ggcaatccta gcgtgaaggc tggtaggatt ttatccccgc tgccatcatg 3960  
 gttcgaccat tgaactgcat cgtcgccgtg tccaaaata tggggattgg caagaacgga 4020  
 gacctaccct ggcctccgct caggaacgag ttcaagtact tccaaagaat gaccacaacc 4080  
 tcttcagtgg aaggtaaaca gaatctggtg attatgggta ggaaaacctg gttctccatt 4140  
 cctgagaaca atcgaccttt aaaggacaga attaatatag ttctcagtag agaactcaaa 4200  
 gaaccaccac gaggagctca ttttcttgcc aaaagtttgg atgatgcctt aagacttatt 4260  
 gaacaaccgg aattggcaag taaagtagac atggtttga tagtcggagg cagttctgtt 4320  
 taccaggaag ccatgaatca accaggccac cttagactct ttgtgacaag gatcatgcag 4380  
 gaatttgaaa gtgacacgtt tttcccagaa attgatttgg ggaaatataa acttctccca 4440  
 gaatacccag gcgtcctctc tgaggtccag gaggaaaaag gcatcaagta taagtttgaa 4500  
 gtctacgaga agaaagacta acaggaagat gctttcaagt tctctgctcc cctcctaaag 4560  
 tcatgcattt ttataagacc atgggacttt tgctggcttt agatcagcct cgactgtgcc 4620  
 ttctagttgc cagccatctg ttgtttgccc ctccccgtg ccttcottga ccctggaagg 4680  
 tgccactccc actgtccttt cctaataaaa tgaggaaatt gcatcgcat gtctgagtag 4740  
 gtgtcattct attctggggg gtggggtggg gcaggacagc aagggggagg attgggaaga 4800  
 caatagcagg catgctgggg atgoggtggg ctctatggaa ccagctgggg ctcgagctac 4860  
 tagctttgct tctcaatttc ttatttgcac aatgagaaaa aaaggaaaat taattttaac 4920  
 accaattcag tagttgattg agcaaatgcg ttgccaaaaa ggatgcttta gagacagtgt 4980  
 tctctgcaca gataaggaca aacattattc agaggagta cccagagctg agactcctaa 5040  
 gccagtgagt ggcacagcat tctagggaga aatatgcttg tcatcacoga agcctgattc 5100  
 cgtagagcca caccttggtg agggccaatc tgctcacaca ggatagagag ggcaggagcc 5160  
 agggcagagc atataagggtg aggtaggatc agttgtcct cacatttgct tctgacatag 5220  
 ttgtgttggg agcttgatc gatcctctat ggttgaacaa gatggattgc acgcaggttc 5280  
 tccggccgct tgggtggaga ggctattcgg ctatgactgg gcacaacaga caatcggctg 5340  
 ctctgatgcc gccgtgttcc ggctgtcagc gcaggggcgc ccggttcttt ttgtcaagac 5400  
 cgacctgtcc ggtgccctga atgaactgca ggacgaggca gcgcggctat cgtggctggc 5460  
 cacgacgggc gttccttgcg cagctgtgct cgacgttgct actgaagcgg gaagggactg 5520  
 gctgctattg ggcaagtgc cggggcagga tctcctgtca tctcaccttg ctctgcca 5580  
 gaaagtatcc atcatggctg atgcaatgcg gcggtgcat acgcttgatc cggctacctg 5640  
 cccattcgac caccaagcga aacatcgcat cgagcgagca cgtactcgga tggaagccgg 5700  
 tcttgtcgat caggatgatc tggacgaaga gcatcagggg ctgcgcccag ccgaactgtt 5760

cgccaggctc aaggcgcgca tgcccgacgg cgaggatctc gtcgtgaccc atggcgatgc 5820  
 ctgcttgccg aatatcatgg tggaaaatgg ccgcttttct ggattcatcg actgtggccg 5880  
 gctgggtgtg gcggaacgct atcaggacat agcgttggct accogtgata ttgctgaaga 5940  
 gcttggcggc gaatgggctg accgcttcct cgtgctttac ggtatcgccg cttcccgatt 6000  
 cgcagcgcat cgccttctat cgccttcttg acgagttctt ctgagcggga ctctggggtt 6060  
 cgaaatgacc gaccaagcga cgcccaacct gccatcacga gatttcgatt ccaccgccgc 6120  
 cttctatgaa aggttgggct tcggaatcgt tttccgggac gccggctgga tgatcctcca 6180  
 gcgcggggat ctcattgctg agttcttcgc ccacccaac ttgtttattg cagcttataa 6240  
 tggttacaaa taaagcaata gcatcacaaa tttcacaaat aaagcatttt tttcactgca 6300  
 ttctagtgtt ggtttgtcca aactcatcaa tctatcttat catgtctgga tcgcggccgc 6360  
 gatcccgctg agagcttggc gtaatcatgg tcatagctgt ttctgtgtg aaattgttat 6420  
 ccgctcacia ttccacacia catacgagcc ggagcataaa gtgtaaagcc tggggtgcct 6480  
 aatgagttag ctaactcaca ttaattgctg tgcgctcact gccgctttc cagtcgggaa 6540  
 acctgtcgtg ccagctgcat taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta 6600  
 ttgggcgctc ttccgcttcc tcgctcactg actcgtcgcg ctcggtcgtt cggctgcggc 6660  
 gagcggatc agctcactca aaggcggtaa tacggttatc cacagaatca ggggataacg 6720  
 caggaaagaa catgtgagca aaaggccagc aaaaggccag gaaccgtaaa aaggccgcgt 6780  
 tgctggcggt tttccatagg ctccgcccc ctgacgagca tcacaaaaat cgacgctcaa 6840  
 gtcagagggt gcgaaacccg acaggactat aaagatacca ggcgtttccc cctggaagct 6900  
 ccctcgtgag ctctcctgtt ccgaccctgc cgcttaccgg atacctgtcc gcctttctcc 6960  
 cttcgggaag cgtggcgctt tctcaatgct cacgctgtag gtatctcagt tcggtgtagg 7020  
 tcgttcgctc caagctgggc tgtgtgcacg aacccccgt tcagcccgac cgctgcgcct 7080  
 tatccggtaa ctatcgtctt gagtccaacc cggttaagaca cgacttatcg cactggcag 7140  
 cagccactgg taacaggatt agcagagcga ggtatgtagg cgggtgctaca gatttcttga 7200  
 agtggtggcc taactacggc tacactagaa ggacagtatt tggatatctgc gctctgctga 7260  
 agccagttac cttcgaaaaa agagttggta gctcttgatc cgcaaacia accaccgctg 7320  
 gtagcgggtg tttttttgtt tgcaagcagc agattacgcg cagaaaaaaa ggatctcaag 7380  
 aagatccttt gatctttct acggggctctg acgctcagt gaacgaaaac tcacgttaag 7440  
 ggattttggt catgagatta tcaaaaagga tcttcaccta gatcctttta aattaaaaat 7500  
 gaagttttta atcaatctaa agtatatatg agtaaacttg gtctgacagt taccaatgct 7560

taatcagtga ggcacctatc tcagcgatct gtctatttcg ttcattccata gttgcctgac 7620  
 tccccgtcgt gtagataact acgatacggg agggcttacc atctggcccc agtgctgcaa 7680  
 tgataccgcg agaccacgc tcaccggctc cagatttatac agcaataaac cagccagccg 7740  
 gaagggccga gcgcagaagt ggtcctgcaa ctttatccgc ctccatccag tctattaatt 7800  
 gttgccggga agctagagta agtagttcgc cagttaatag ttgcgcaac gttgttgcca 7860  
 ttgctacagg catcgtgggtg tcacgctcgt cgtttggtat ggcttcattc agctccgggt 7920  
 cccaacgatc aaggcgagtt acatgatccc ccatgttgtg caaaaaagcg gttagctcct 7980  
 tcggctcctcc gatcgttgtc agaagtaagt tggccgcagt gttatcactc atgggttatgg 8040  
 cagcactgca taattctctt actgtcatgc catccgtaag atgcttttct gtgactgggtg 8100  
 agtactcaac caagtcattc tgagaatagt gtatgcggcg accgagttgc tcttgcccgg 8160  
 cgtcaatacg ggataatacc gcgccacata gcagaacttt aaaagtgtc atcattggaa 8220  
 aacgttcttc ggggcgaaaa ctctcaagga tcttaccgct gttgagatcc agttcgatgt 8280  
 aaccactcg tgcacccaac tgatcttcag catcttttac tttcaccagc gtttctgggt 8340  
 gagcaaaaac aggaaggcaa aatgcgcga aaaagggaa aagggcgaca cggaaatgtt 8400  
 gaatactcat actcttctt tttcaatatt attgaagcat ttatcaggggt tattgtctca 8460  
 tgagcggata catatttgaa tgtatttaga aaaataaaca aataggggtt ccgcgcacat 8520  
 ttccccgaaa agtgccacct 8540

&lt;210&gt; 58

&lt;211&gt; 9209

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;400&gt; 58

gacgtcgcgg ccgctctagg cctccaaaaa agcctcctca ctacttctgg aatagctcag 60  
 aggcgcaggc ggctcggcc tctgcataaa taataaaat tagtcagcca tgcattgggc 120  
 ggagaatggg cggaactggg cggagttagg ggcgggatgg gcggagttag gggcgggact 180  
 atggttgctg actaattgag atgcatgctt tgcatacttc tgctgctgg ggagcctggg 240  
 gactttccac acctgggtgc tgactaattg agatgcatgc tttgcatact tctgctgct 300  
 ggggagcctg gggactttcc acaccctaac tgacacacat tccacagaat taattcccct 360  
 agttattaat agtaatcaat tacggggcca ttagttcata gccatataat ggagttccgc 420  
 gttacataac ttacggtaaa tggcccgctt ggctgaccgc ccaacgaccc ccgcccattg 480  
 acgtcaataa tgacgtatgt tcccatagta acgccaatag ggactttcca ttgacgtcaa 540  
 tgggtggact atttacggta aactgccac ttggcagtac atcaagtgt tcatatgcca 600  
 agtaaccccc ctattgacgt caatgacggt aatggccc cctggcatta tgcccagtac 660

atgacottat gggactttcc tacttggcag tacatctacg tattagtcac cgctattacc 720  
 atgggtgatgc ggttttggca gtacatcaat gggcgtggat accggtttga ctcacgcgga 780  
 tttccaagtc tccaccccat tgacgtcaat gggagtttgt tttggcacca aaatcaacgg 840  
 gactttccaa aatgtcgtaa caactccgcc ccattgacgc aaatgggcgg taggcgtgta 900  
 cggtgggagg tctatataag cagagctggg tacgtgaacc gtcagatcgc ctggagacgc 960  
 catcacagat ctctcactat ggattttcag gtgcagatta tcagcttcct gctaatacgt 1020  
 gcttcagtca taatgtccag aggacaaatt gttctctccc agtctccagc aatcctgtct 1080  
 gcatctccag gggagaaggt cacaatgact tgcagggcca gctcaagtgt aagttacatc 1140  
 cactggttcc agcagaagcc aggatcctcc cccaaaccct ggatttatgc cacatccaac 1200  
 ctggcttctg gagtccctgt tgcgttcagt ggcagtgggt ctgggacttc ttactctctc 1260  
 acaatcagca gagtggaggc tgaagatgct gccacttatt actgccagca gtggactagt 1320  
 aaccacacca cgttcggagg ggggaccaag ctggaaatca aacgtacggt ggctgcacca 1380  
 tctgtcttca tcttcccgcc atctgatgag cagttgaaat ctggaactgc ctctgttgtg 1440  
 tgctgtctga ataacttcta tcccagagag gccaaagtac agtggaaggt ggataacgcc 1500  
 ctccaatcgg gtaactccca ggagagtgtc acagagcagg acagcaagga cagcacctac 1560  
 agcctcagca gcaccctgac gctgagcaaa gcagactacg agaaacacaa agtctacgcc 1620  
 tgcaagtca cccatcaggg cctgagctcg cccgtcacia agagcttcaa caggggagag 1680  
 tgttgaattc agatccgtta acggttacca actacctaga ctggattcgt gacaacatgc 1740  
 ggccgtgata tctacgtatg atcagcctcg actgtgcctt ctagttgcca gccatctgtt 1800  
 gtttgcctc ccccgctgcc ttcttgacc ctggaaggtg ccaactccac tgtcctttcc 1860  
 taataaaatg aggaaattgc atcgattgt ctgagtaggt gtcattctat tctgggggggt 1920  
 ggggtggggc aggacagcaa gggggaggat tgggaagaca atagcaggca tgctggggat 1980  
 gcggtgggct ctatggaacc agctggggct cgacagctat gccaagtacg cccctatttg 2040  
 acgtcaatga cggtaaatgg cccgcctggc attatgccca gtacatgacc ttatgggact 2100  
 ttcctacttg gcagtacatc tacgtattag tcatcgctat taccatgggt atgcggtttt 2160  
 ggcagtacat caatgggcgt ggatagcggg ttgactcacg gggatttcca agtctccacc 2220  
 ccattgacgt caatgggagt ttgttttggc accaaaatca acgggacttt ccaaaatgtc 2280  
 gtaacaactc cgccccattg acgcaaatgg gcggtaggcg tgtacgggtg gaggtctata 2340  
 taagcagagc tgggtacgtc ctcacattca gtgatcagca ctgaacacag acccgctcag 2400  
 atgggttga gcctcatctt gctcttctt gtcgctgttg ctacgcgtgt cctgtcccag 2460

gtacaactgc agcagcctgg ggctgagctg gtgaagcctg gggcctca<sup>8</sup>gt gaagatg<sup>9</sup>g<sup>10</sup>cc 2520  
 tgcaaggctt ctggctacac atttaccagt tacaatatgc actgggtaaa acagacacct 2580  
 ggtcggggcc tggaatggat tggagctatt tatcccgaa atgggtgatac ttcctacaat 2640  
 cagaagttca aaggcaaggc cacattgact gcagacaaat cctccagcac agcctacatg 2700  
 cagctcagca gcctgacatc tgaggactct gcggtctatt actgtgcaag atcgacttac 2760  
 tacggcgggtg actggtactt caatgtctgg ggcgagggga ccacg<sup>11</sup>gtcac cgtctctgca 2820  
 gctagcacca agggcccatc ggtcttcccc ctggcaccct cctccaagag cacctctggg 2880  
 ggcacagcgg ccctgggctg cctgggtcaag gactacttcc ccgaaccggg gacgggtgtcg 2940  
 tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtccca 3000  
 ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg caccagacc 3060  
 tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagaa agcagagccc 3120  
 aaatcttgtg aaaaaactca cacatgccca ccgtg<sup>12</sup>ccag cacctgaact cctgggggga 3180  
 ccgtcagtct tcctcttccc cccaaaacc aaggacacc tcatgatctc ccggaccct 3240  
 gaggtcacat gcgtgggtgt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 3300  
 tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 3360  
 agcacgtacc gtgtggtcag cgtcctcacc gtcctgcacc aggactggct gaatggcaag 3420  
 gagtacaagt gcaagggtct caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 3480  
 aaagccaaag ggcagccccg agaaccacag gtgtacacc tgccccatc ccgggatgag 3540  
 ctgaccaaga accaggtcag cctgacctgc ctgggtcaaag gcttctatcc cagcgacatc 3600  
 gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccg<sup>13</sup>tg 3660  
 ctggactccg acggctcctt ctctctctac agcaagctca ccgtggacaa gagcagggtg 3720  
 cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 3780  
 cagaagagcc tctccctgtc tccgggtaaa tgaggatccg ttaacggtta ccaactacct 3840  
 agactggatt cgtgacaaca tgcggccgtg atatctacgt atgacagcc tcgactgtgc 3900  
 cttctagttg ccagccatct gttgtttgcc cctccccctg gccttcttg accctggaag 3960  
 gtgccactcc cactgtcctt tcctaataaa atgaggaaat tgcacgcat tgtctgagta 4020  
 ggtgtcattc tattctgggg ggtgggggtg ggcaggacag caagggggag gattgggaag 4080  
 acaatagcag gcatgctggg gatgcggtg gctctatgga accagctggg gctcgacacg 4140  
 gctggatctc ccgatcccca gctttgcttc tcaatttctt atttgcataa tgagaaaaaa 4200  
 aggaaaatta attttaacac caattcagta gttgattgag caaatgcgtt gccaaaaagg 4260  
 atgctttaga gacagtgttc tctgcacaga taaggacaaa cattattcag agggagtacc 4320



cagagctgag actcctaagc cagtgagtgg cacagcattc tagggagaaa tatgcttgtc 4380  
 atcaccgaag cctgattccg tagagccaca ccttggttaag ggccaatctg ctcacacagg 4440  
 atagagaggg caggagccag ggcagagcat ataagggtgag gtaggatcag ttgctcctca 4500  
 catttgcttc tgacatagtt gtgttgggag cttggatagc ttggacagct cagggctgcg 4560  
 atttcgcgcc aaacttgacg gcaatcctag cgtgaaggct ggtaggattt tatccccgct 4620  
 gccatcatgg ttcgaccatt gaactgcacg gtcgccgtgt cccaaaatat ggggattggc 4680  
 aagaacggag acctaccctg gcctccgctc aggaacgagt tcaagtactt ccaaagaatg 4740  
 accacaacct cttcagtggg aggtaaacag aatctggtga ttatgggtag gaaaacctgg 4800  
 ttctccattc ctgagaagaa tcgaccttta aaggacagaa ttaatatagt tctcagtaga 4860  
 gaactcaaag aaccaccacg aggagctcat tttcttgcca aaagtttggg tgatgcctta 4920  
 agacttattg aacaaccgga attggcaagt aaagtagaca tggtttggat agtcggaggc 4980  
 agttctgttt accaggaagc catgaatcaa ccaggccacc ttagactctt tgtgacaagg 5040  
 atcatgcagg aatttgaaag tgacacgttt ttcccagaaa ttgatttggg gaaatataaa 5100  
 cttctccag aatacccagg cgtcctctct gaggtccagg aggaaaaagg catcaagtat 5160  
 aagtttgaag tctacgagaa gaaagactaa caggaagatg ctttcaagtt ctctgctccc 5220  
 ctctaaagc tatgcatttt tataagacca tgggaactttt gctggcttta gatcagcctc 5280  
 gactgtgcct tctagttgcc agccatctgt tgtttgcccc tccccgtgc cttccttgac 5340  
 cctggaaggt gccactocca ctgtcctttc ctaataaaat gaggaattg catcgcattg 5400  
 tctgagtagg tgtcattcta ttctgggggg tggggtgggg caggacagca agggggagga 5460  
 ttgggaagac aatagcaggc atgctgggga tgcggtgggc tctatggaac cagctggggc 5520  
 tcgagctact agctttgctt ctcaatttct tatttgcata atgagaaaaa aaggaaaatt 5580  
 aattttaaca ccaattcagt agttgattga gcaaatgcgt tgccaaaaag gatgctttag 5640  
 agacagtgtt ctctgcacag ataaggacaa acattattca gagggagtac ccagagctga 5700  
 gactcctaag ccagtgagtg gcacagcatt ctagggagaa atatgcttgt catcacogaa 5760  
 gcctgattcc gtagagccac accttggtta gggccaatct gctcacacag gatagagagg 5820  
 gcaggagcca gggcagagca tataaggtga ggtaggatca gttgctcctc acatttgctt 5880  
 ctgacatagt tgtgttggga gcttgatcg atcctctatg gttgaacaag atggattgca 5940  
 cgcaggttct ccggccgctt ggggtggagag gctattcggc tatgactggg cacaacagac 6000  
 aatcggtgc tctgatgccg ccgtgttccg gctgtcagcg caggggcgcc cggttctttt 6060  
 tgtcaagacc gacctgtccg gtgcctgaa tgaactgcag gacgaggcag cgcggctatc 6120

gtggctggcc acgacgggcg ttccttgccg agctgtgctc gacgttggtca ctgaagcggg 6180  
 aagggactgg ctgctattgg gcgaagtgcc ggggcaggat ctcctgtcat ctcaccttgc 6240  
 tcctgccgag aaagtatcca tcatggctga tgcaatgcgg cggctgcata cgcttgatcc 6300  
 ggctacctgc ccattcgacc accaagcgaa acatcgcatc gagcgagcac gtactcggat 6360  
 ggaagccggt cttgtcgatc aggatgatct ggacgaagag catcaggggc tcgcgccagc 6420  
 cgaactgttc gccaggctca aggcgcgcat gcccgacggc gaggatctcg tcgtgaccca 6480  
 tggcgatgcc tgcttgccga atatcatggt ggaaaatggc cgcttttctg gattcatcga 6540  
 ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata gcgttggtta cccgtgatat 6600  
 tgctgaagag cttggcggcg aatgggctga ccgcttcctc gtgctttaag gtatcgccgc 6660  
 tcccgattcg cagcgcatcg ccttctatcg ccttcttgac gagttcttct gagcgggact 6720  
 ctggggttcg aaatgaccga ccaagcgacg cccaacctgc catcacgaga tttcgattcc 6780  
 accgcgcct tctatgaaag gttgggcttc ggaatcgttt tccgggacgc cggctggatg 6840  
 atcctccagc gcggggatct catgctggag ttcttcgccc accccaactt gtttattgca 6900  
 gcttataatg gttacaaata aagcaatagc atcacaaatt tcacaaataa agcatttttt 6960  
 tcaactgcatt ctagttgtgg tttgtccaaa ctcacaaatc tatcttatca tgtctggatc 7020  
 gcggccgcga tcccgctcag agcttgccgt aatcatggtc atagctgttt cctgtgtgaa 7080  
 attgttatcc gtcacaaatt ccacacaaca tacgagccgg aagcataaag tgtaaagcct 7140  
 ggggtgccta atgagtgage taactacat taattgcgtt gcgctcactg cccgctttcc 7200  
 agtcgggaaa cctgtcgtgc cagctgcatt aatgaatcgg ccaacgcgcg gggagaggcg 7260  
 gtttgcgat tgggcgctct tccgcttcc cgtcactga ctcgctgcgc tcggtcgttc 7320  
 ggctgcggcg agcggtatca gctcactcaa aggcggtaat acggttatcc acagaatcag 7380  
 gggataacgc aggaagaac atgtgagcaa aaggcagca aaaggccagg aaccgtaaaa 7440  
 aggcgcgctt gctggcgctt ttccataggc tccgcccccc tgacgagcat cacaaaaatc 7500  
 gacgctcaag tcagaggtgg cgaaaccgga caggactata aagataccag gcgtttcccc 7560  
 ctggaagctc cctcgtgcgc tctcctgttc cgaccctgcc gcttaccgga tacctgtccg 7620  
 cctttctccc ttcgggaagc gtggcgcttt ctcaatgctc acgctgtagg tatctcagtt 7680  
 cggtgtaggt cgctcgtcc aagctgggct gtgtgcacga acccccgctt cagcccgacc 7740  
 gctgcgcctt atccggtaac tatcgtcttg agtccaaccc ggtaagacac gacttatcgc 7800  
 cactggcagc agccactggt aacaggatta gcagagcgag gtatgtaggc ggtgctacag 7860  
 agttcttgaa gtggtggcct aactacggct acactagaag gacagtattt ggtatctgcg 7920  
 ctctgctgaa gccagttacc ttcggaaaaa gagttggtag ctcttgatcc ggcaaaaaa 7980

ccaccgctgg tagcgggtgg ttttttgttt gcaagcagca gattacgcgc agaaaaaaag 8040  
 gatctcaaga agatcctttg atcttttcta cgggggtctga cgctcagtgg aacgaaaact 8100  
 cacgttaagg gattttggtc atgagattat caaaaaggat cttcacctag atccttttaa 8160  
 attaaaaatg aagttttaaa tcaatctaaa gtatatatga gtaaacttgg tctgacagtt 8220  
 accaatgctt aatcagttag gcacctatct cagcgatctg tctatttcgt tcatccatag 8280  
 ttgcctgact ccccgctcgt tagataacta cgatacggga gggcttacca tctggcccca 8340  
 gtgctgcaat gataccgcga gaccacgct caccggtcc agatttatca gcaataaacc 8400  
 agccagccgg aagggccgag cgcagaagtg gtcctgcaac tttatccgcc tccatccagt 8460  
 ctattaattg ttgccgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg 8520  
 ttgttgccat tgctacaggc atcgtggtgt cagctcgtc gtttggtatg gcttcattca 8580  
 gctccggttc ccaacgatca aggcgagta catgatccc catgttgtgc aaaaaagcgg 8640  
 ttagctcctt cggctcctccg atcgttgtca gaagtaagtt ggccgcagtg ttatcactca 8700  
 tggttatggc agcactgcat aattctctta ctgtcatgcc atccgtaaga tgcttttctg 8760  
 tgactgggtga gtactcaacc aagtcattct gagaatagt tatgcggcga ccgagttgct 8820  
 cttgcccggc gtcaatacgg gataataccg cgccacatag cagaacttta aaagtgtca 8880  
 tcattggaaa acgttcttcg gggcgaaaac tctcaaggat cttaccgctg ttgagatcca 8940  
 gttcgatgta acccactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg 9000  
 tttctgggtg agcaaaaaca ggaaggcaaa atgccgcaaa aaagggaata agggcgacac 9060  
 ggaaatgttg aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcaggggtt 9120  
 attgtctcat gagcggatac atatttgaat gtatttagaa aaataaaca ataggggttc 9180  
 cgcgcacatt tccccgaaa gtgccacct 9209

&lt;210&gt; 59

&lt;211&gt; 384

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;400&gt; 59

atggattttc aggtgcagat tatcagcttc ctgctaata gtgcttcagt cataatgtcc 60  
 agagggcaaa ttgttctctc ccagtctcca gcaatcctgt ctgcatctcc aggggagaag 120  
 gtcacaatga cttgcagggc cagctcaagt gtaagttaca tccactgggt ccagcagaag 180  
 ccaggatcct ccccaaacc ctggatttat gccacatcca acctggcttc tggagtccct 240  
 gttcgcttca gtggcagtgg gtctgggact tcttactctc tcacaatcag cagagtggag 300  
 gctgaagatg ctgccactta ttactgccag cagtggacta gtaaccacc caggttcgga 360

ggggggacca agctggaaat caaa

384

<210> 60  
 <211> 128  
 <212> PRT  
 <213> Mus musculus

<400> 60  
 Met Asp Phe Gln Val Gln Ile Ile Ser Phe Leu Leu Ile Ser Ala Ser  
 1 5 10 15  
 Val Ile Met Ser Arg Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Ile  
 20 25 30  
 Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Arg Ala Ser  
 35 40 45  
 Ser Ser Val Ser Tyr Ile His Trp Phe Gln Gln Lys Pro Gly Ser Ser  
 50 55 60  
 Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro  
 65 70 75 80  
 Val Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile  
 85 90 95  
 Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp  
 100 105 110  
 Thr Ser Asn Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys  
 115 120 125

<210> 61  
 <211> 420  
 <212> DNA  
 <213> Mus musculus

<400> 61  
 atgggttgga gcctcatctt gctcttcctt gtcgctgttg ctacgcgtgt cctgtcccag 60  
 gtacaactgc agcagcctgg ggctgagctg gtgaagcctg gggcctcagt gaagatgtcc 120  
 tgcaaggcctt ctggctacac atttaccagt tacaatatgc actgggtaaa acagacacct 180  
 ggtcggggcc tggaatggat tggagctatt tatcccggaa atggtgatac ttcctacaat 240  
 cagaagttca aaggcaaggc cacattgact gcagacaaat cctccagcac agcctacatg 300  
 cagctcagca gcctgacatc tgaggactct gcgggtctatt actgtgcaag atcgacttac 360  
 tacggcgggtg actggtactt caatgtcttg ggcgagggga ccaagggtcac cgtctctgca 420

<210> 62  
 <211> 140  
 <212> PRT  
 <213> Mus musculus

<400> 62  
 Met Gly Trp Ser Leu Ile Leu Leu Phe Leu Val Ala Val Ala Thr Arg  
 1 5 10 15

Val Leu Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys  
 20 25 30

Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe  
 35 40 45

Thr Ser Tyr Asn Met His Trp Val Lys Gln Thr Pro Gly Arg Gly Leu  
 50 55 60

Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn  
 65 70 75 80

Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser  
 85 90 95

Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val  
 100 105 110

Tyr Tyr Cys Ala Arg Ser Thr Tyr Tyr Gly Gly Asp Trp Tyr Phe Asn  
 115 120 125

Val Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ala  
 130 135 140

<210> 63  
 <211> 1395  
 <212> DNA  
 <213> Homo sapiens

<400> 63  
 atgtattcca atgtgatagg aactgtaacc tctggaaaaa ggaaggttta tcttttgtcc 60  
 ttgctgctca ttggcttctg ggactgcgtg acctgtcacg ggagccctgt ggacatctgc 120  
 acagccaagc cgcgggacat tcccatgaat cccatgtgca tttaccgctc cccggagaag 180  
 aaggcaactg aggatgaggg ctcaaacag aagatcccgg aggccaccaa ccggcgtgtc 240  
 tgggaactgt ccaaggccaa ttcccgttt gctaccactt tctatcagca cctggcagat 300  
 tccaagaatg acaatgataa cattttcctg tcaccctga gtatctccac ggcttttgct 360  
 atgaccaagc tgggtgcctg taatgacacc ctccagcaac tgatggaggt atttaagttt 420  
 gacaccatat ctgagaaaac atctgatcag atccacttct tctttgcaa actgaactgc 480  
 cgactctatc gaaaagccaa caaatcctcc aagttagtat cagccaatcg cctttttgga 540  
 gacaaatccc ttaccttcaa tgagacctac caggacatca gtgagttggt atatggagcc 600  
 aagctccagc ccctggactt caaggaaaat gcagagcaat ccagagcggc catcaacaaa 660  
 tgggtgtcca ataagaccga aggccgaatc accgatgtca ttccctcgga agccatcaat 720  
 gagctcactg ttctggtgct ggtaacacc atttacttca agggcctgtg gaagtcaaag 780  
 ttcagccctg agaacacaag gaaggaaactg ttctacaagg ctgatggaga gtcgtgttca 840  
 gcatctatga tgtaccagga aggcaagttc cgttatcggc gcgtggctga aggcaccag 900

gtgcttgagt tgcccttcaa aggtgatgac atcaccatgg tcctcatctt gcccaagcct 960  
 gagaagagcc tggccaaggt ggagaaggaa ctcaccccag aggtgctgca ggagtggctg 1020  
 gatgaattgg aggagatgat gctggtggtc cacatgcccc gcttccgcat tgaggacggc 1080  
 ttcagtttga aggagcagct gcaagacatg ggccttgctg atctgttcag ccctgaaaag 1140  
 tccaaactcc caggtattgt tgcagaaggc cgagatgacc tctatgtctc agatgcattc 1200  
 cataaggcat ttcttgaggt aaatgaagaa ggcagtgaag cagctgcaag taccgctgtt 1260  
 gtgattgctg gccgttcgct aaacccaac agggtgactt tcaaggccaa caggcctttc 1320  
 ctggttttta taagagaagt tcctctgaac actattatct tcatgggcag agtagccaac 1380  
 ccttggttta agtaa 1395

<210> 64  
 <211> 464  
 <212> PRT  
 <213> Homo sapiens

<400> 64  
 Met Tyr Ser Asn Val Ile Gly Thr Val Thr Ser Gly Lys Arg Lys Val  
 1 5 10 15  
 Tyr Leu Leu Ser Leu Leu Leu Ile Gly Phe Trp Asp Cys Val Thr Cys  
 20 25 30  
 His Gly Ser Pro Val Asp Ile Cys Thr Ala Lys Pro Arg Asp Ile Pro  
 35 40 45  
 Met Asn Pro Met Cys Ile Tyr Arg Ser Pro Glu Lys Lys Ala Thr Glu  
 50 55 60  
 Asp Glu Gly Ser Glu Gln Lys Ile Pro Glu Ala Thr Asn Arg Arg Val  
 65 70 75 80  
 Trp Glu Leu Ser Lys Ala Asn Ser Arg Phe Ala Thr Thr Phe Tyr Gln  
 85 90 95  
 His Leu Ala Asp Ser Lys Asn Asp Asn Asp Asn Ile Phe Leu Ser Pro  
 100 105 110  
 Leu Ser Ile Ser Thr Ala Phe Ala Met Thr Lys Leu Gly Ala Cys Asn  
 115 120 125  
 Asp Thr Leu Gln Gln Leu Met Glu Val Phe Lys Phe Asp Thr Ile Ser  
 130 135 140  
 Glu Lys Thr Ser Asp Gln Ile His Phe Phe Phe Ala Lys Leu Asn Cys  
 145 150 155 160  
 Arg Leu Tyr Arg Lys Ala Asn Lys Ser Ser Lys Leu Val Ser Ala Asn  
 165 170 175  
 Arg Leu Phe Gly Asp Lys Ser Leu Thr Phe Asn Glu Thr Tyr Gln Asp  
 180 185 190  
 Ile Ser Glu Leu Val Tyr Gly Ala Lys Leu Gln Pro Leu Asp Phe Lys

195	200	205
Glu Asn Ala Glu Gln Ser Arg Ala Ala Ile Asn Lys Trp Val Ser Asn 210 215 220		
Lys Thr Glu Gly Arg Ile Thr Asp Val Ile Pro Ser Glu Ala Ile Asn 225 230 235 240		
Glu Leu Thr Val Leu Val Leu Val Asn Thr Ile Tyr Phe Lys Gly Leu 245 250 255		
Trp Lys Ser Lys Phe Ser Pro Glu Asn Thr Arg Lys Glu Leu Phe Tyr 260 265 270		
Lys Ala Asp Gly Glu Ser Cys Ser Ala Ser Met Met Tyr Gln Glu Gly 275 280 285		
Lys Phe Arg Tyr Arg Arg Val Ala Glu Gly Thr Gln Val Leu Glu Leu 290 295 300		
Pro Phe Lys Gly Asp Asp Ile Thr Met Val Leu Ile Leu Pro Lys Pro 305 310 315 320		
Glu Lys Ser Leu Ala Lys Val Glu Lys Glu Leu Thr Pro Glu Val Leu 325 330 335		
Gln Glu Trp Leu Asp Glu Leu Glu Glu Met Met Leu Val Val His Met 340 345 350		
Pro Arg Phe Arg Ile Glu Asp Gly Phe Ser Leu Lys Glu Gln Leu Gln 355 360 365		
Asp Met Gly Leu Val Asp Leu Phe Ser Pro Glu Lys Ser Lys Leu Pro 370 375 380		
Gly Ile Val Ala Glu Gly Arg Asp Asp Leu Tyr Val Ser Asp Ala Phe 385 390 395 400		
His Lys Ala Phe Leu Glu Val Asn Glu Glu Gly Ser Glu Ala Ala Ala 405 410 415		
Ser Thr Ala Val Val Ile Ala Gly Arg Ser Leu Asn Pro Asn Arg Val 420 425 430		
Thr Phe Lys Ala Asn Arg Pro Phe Leu Val Phe Ile Arg Glu Val Pro 435 440 445		
Leu Asn Thr Ile Ile Phe Met Gly Arg Val Ala Asn Pro Cys Val Lys 450 455 460		

<210> 65  
 <211> 1962  
 <212> DNA  
 <213> Homo sapiens

<400> 65  
 atgcgtcccc tgcgc'ccccg cgccgcgctg ctggcgctcc tggcctcgct cctggccgcg 6  
 ccccggtgg ccccgccga ggccccgcac ctggtgcagg tggacgcggc ccgcgcgctg 12  
 tggccctgc ggcgcttctg gaggagcaca ggcttctgcc ccccgctgcc acacagccag 18

gctgaccagt acgtcctcag ctgggaccag cagctcaacc tcgcctatgt gggcgccgtc 240  
 cctcacccgc gcatcaagca ggtccggacc cactggctgc tggagcttgt caccaccagg 300  
 ggggtccactg gacggggcct gagctacaac ttcacccacc tggacgggta cttggacctt 360  
 ctcagggaga accagctcct ccaggggttt gagctgatgg gcagcgccctc gggccacttc 420  
 actgactttg aggacaagca gcaggtgttt gagtggaaagg acttgggtctc cagcctggcc 480  
 aggagataca tcggtaggta cggactggcg catgtttcca agtggaaactt cgagacgtgg 540  
 aatgagccag accaccacga ctttgacaac gtctccatga ccatgcaagg cttcctgaac 600  
 tactacgatg cctgctcgga gggctctgcg gccgccagcc ccgccctgcg gctgggagggc 660  
 cccggcgact ccttcacac ccaccgcga tcccgcgtga gctggggcct cctgcgccac 720  
 tgccacgacg gtaccaactt cttcactggg gagggggcg tgccgctgga ctacatctcc 780  
 ctccacagga aggtgctgcg cagctccatc tccatcctgg agcaggagaa ggtcgtcgcg 840  
 cagcagatcc ggcagctctt cccaagttc gcggacacc ccatttaca cgacgagggcg 900  
 gacccgctgg tgggtgtgtc cctgccacag ccgtggaggg cgacgtgac ctacgcggcc 960  
 atggtgtgtga aggtcatcgc gcagcatcag aacctgctac tggccaacac cacctccgcc 1020  
 ttcccctacg cgctcctgag caacgacaat gccttctga gctaccacc gcaccccttc 1080  
 gcgcagcgca cgctcaccgc gcgcttcag gtcaacaaca cccgcccgcc gcacgtgacg 1140  
 ctgttgcgca agccggtgct cacggccatg gggctgctgg cgctgctgga tgaggagcag 1200  
 ctctggggccg aagtgtcgca ggccgggacc gtccctggaca gcaaccacac ggtgggcgtc 1260  
 ctggccagcg cccaccgccc ccagggcccgc gccgacgctt ggcgcgcgcg ggtgctgatc 1320  
 tacgcgagcg acgacacccg cggccacccc aacgcgagcg tcgcggtgac cctgcggctg 1380  
 cgcggggtgc ccccgggccc gggcctggtc tacgtcacgc gctacctgga caacgggtc 1440  
 tgcagccccg acggcgagtg gcggcgccct ggccggcccc tcttcccac ggcagagcag 1500  
 ttccggcgca tgcgcgcggc tgaggacccg gtggcgcgg cgccccgcc cttaccgcc 1560  
 ggcggccgcc tgacctgcg cccgcgctg cggtgccgt cgcttttgc ggtgcacgtg 1620  
 tgtgcgcgcc ccgagaagcc gccgggagc gtcacgcggc tccgcgcct gccctgacc 1680  
 caagggcagc tggttctggt ctggtcggat gaacacgtgg gctccaagt cctgtggaca 1740  
 tacgagatcc agttctctca ggacggtaag gcgtacacc cggtcagcag gaagccatcg 1800  
 accttaacc tctttgtgtt cagcccagac acaggtgctg tctctggctc ctaccgagtt 1860  
 cgagccctgg actactgggc ccgaccagg cccttctcgg accctgtgcc gtacctggag 1920  
 gtccctgtgc caagagggcc cccatcccc ggcaatccat ga 1962



<210> 66  
 <211> 653  
 <212> PRT  
 <213> Homo sapiens

<400> 66

```

Met Arg Pro Leu Arg Pro Arg Ala Ala Leu Leu Ala Leu Leu Ala Ser
1          5          10          15

Leu Leu Ala Ala Pro Pro Val Ala Pro Ala Glu Ala Pro His Leu Val
20          25          30

Gln Val Asp Ala Ala Arg Ala Leu Trp Pro Leu Arg Arg Phe Trp Arg
35          40          45

Ser Thr Gly Phe Cys Pro Pro Leu Pro His Ser Gln Ala Asp Gln Tyr
50          55          60

Val Leu Ser Trp Asp Gln Gln Leu Asn Leu Ala Tyr Val Gly Ala Val
65          70          75          80

Pro His Arg Gly Ile Lys Gln Val Arg Thr His Trp Leu Leu Glu Leu
85          90          95

Val Thr Thr Arg Gly Ser Thr Gly Arg Gly Leu Ser Tyr Asn Phe Thr
100         105         110

His Leu Asp Gly Tyr Leu Asp Leu Leu Arg Glu Asn Gln Leu Leu Pro
115         120         125

Gly Phe Glu Leu Met Gly Ser Ala Ser Gly His Phe Thr Asp Phe Glu
130         135         140

Asp Lys Gln Gln Val Phe Glu Trp Lys Asp Leu Val Ser Ser Leu Ala
145         150         155         160

Arg Arg Tyr Ile Gly Arg Tyr Gly Leu Ala His Val Ser Lys Trp Asn
165         170         175

Phe Glu Thr Trp Asn Glu Pro Asp His His Asp Phe Asp Asn Val Ser
180         185         190

Met Thr Met Gln Gly Phe Leu Asn Tyr Tyr Asp Ala Cys Ser Glu Gly
195         200         205

Leu Arg Ala Ala Ser Pro Ala Leu Arg Leu Gly Gly Pro Gly Asp Ser
210         215         220

Phe His Thr Pro Pro Arg Ser Pro Leu Ser Trp Gly Leu Leu Arg His
225         230         235         240

Cys His Asp Gly Thr Asn Phe Phe Thr Gly Glu Ala Gly Val Arg Leu
245         250         255

Asp Tyr Ile Ser Leu His Arg Lys Gly Ala Arg Ser Ser Ile Ser Ile
260         265         270

Leu Glu Gln Glu Lys Val Val Ala Gln Gln Ile Arg Gln Leu Phe Pro
275         280         285

Lys Phe Ala Asp Thr Pro Ile Tyr Asn Asp Glu Ala Asp Pro Leu Val

```

290					295					300					
Gly	Trp	Ser	Leu	Pro	Gln	Pro	Trp	Arg	Ala	Asp	Val	Thr	Tyr	Ala	Ala
305					310					315					320
Met	Val	Val	Lys	Val	Ile	Ala	Gln	His	Gln	Asn	Leu	Leu	Leu	Ala	Asn
				325					330					335	
Thr	Thr	Ser	Ala	Phe	Pro	Tyr	Ala	Leu	Leu	Ser	Asn	Asp	Asn	Ala	Phe
			340					345					350		
Leu	Ser	Tyr	His	Pro	His	Pro	Phe	Ala	Gln	Arg	Thr	Leu	Thr	Ala	Arg
		355					360					365			
Phe	Gln	Val	Asn	Asn	Thr	Arg	Pro	Pro	His	Val	Gln	Leu	Leu	Arg	Lys
	370					375					380				
Pro	Val	Leu	Thr	Ala	Met	Gly	Leu	Leu	Ala	Leu	Leu	Asp	Glu	Glu	Gln
385					390					395					400
Leu	Trp	Ala	Glu	Val	Ser	Gln	Ala	Gly	Thr	Val	Leu	Asp	Ser	Asn	His
			405					410						415	
Thr	Val	Gly	Val	Leu	Ala	Ser	Ala	His	Arg	Pro	Gln	Gly	Pro	Ala	Asp
		420					425					430			
Ala	Trp	Arg	Ala	Ala	Val	Leu	Ile	Tyr	Ala	Ser	Asp	Asp	Thr	Arg	Ala
		435					440					445			
His	Pro	Asn	Arg	Ser	Val	Ala	Val	Thr	Leu	Arg	Leu	Arg	Gly	Val	Pro
	450					455					460				
Pro	Gly	Pro	Gly	Leu	Val	Tyr	Val	Thr	Arg	Tyr	Leu	Asp	Asn	Gly	Leu
465				470						475					480
Cys	Ser	Pro	Asp	Gly	Glu	Trp	Arg	Arg	Leu	Gly	Arg	Pro	Val	Phe	Pro
			485						490					495	
Thr	Ala	Glu	Gln	Phe	Arg	Arg	Met	Arg	Ala	Ala	Glu	Asp	Pro	Val	Ala
		500					505					510			
Ala	Ala	Pro	Arg	Pro	Leu	Pro	Ala	Gly	Gly	Arg	Leu	Thr	Leu	Arg	Pro
		515					520					525			
Ala	Leu	Arg	Leu	Pro	Ser	Leu	Leu	Val	His	Val	Cys	Ala	Arg	Pro	
	530					535					540				
Glu	Lys	Pro	Pro	Gly	Gln	Val	Thr	Arg	Leu	Arg	Ala	Leu	Pro	Leu	Thr
545				550						555					560
Gln	Gly	Gln	Leu	Val	Leu	Val	Trp	Ser	Asp	Glu	His	Val	Gly	Ser	Lys
			565						570					575	
Cys	Leu	Trp	Thr	Tyr	Glu	Ile	Gln	Phe	Ser	Gln	Asp	Gly	Lys	Ala	Tyr
		580					585						590		
Thr	Pro	Val	Ser	Arg	Lys	Pro	Ser	Thr	Phe	Asn	Leu	Phe	Val	Phe	Ser
		595					600					605			
Pro	Asp	Thr	Gly	Ala	Val	Ser	Gly	Ser	Tyr	Arg	Val	Arg	Ala	Leu	Asp
	610					615					620				

Tyr Trp Ala Arg Pro Gly Pro Phe Ser Asp Pro Val Pro Tyr Leu Glu  
625 630 635 640

Val Pro Val Pro Arg Gly Pro Pro Ser Pro Gly Asn Pro  
645 650

<210> 67  
<211> 1290  
<212> DNA  
<213> Homo sapiens

<400> 67  
atgcagctga ggaaccaga actacatctg ggctgcgcgc ttgcgcttcg cttcctggcc 60  
ctcgtttcct gggacatccc tggggctaga gcaactggaca atggattggc aaggacgcct 120  
accatgggct ggctgcactg ggagcgcttc atgtgcaacc ttgactgcca ggaagagcca 180  
gattcctgca tcagtggaga gctcttcatt gagatggcag agctcatggg ctcagaaggg 240  
tggaaggatg caggttatga gtacctctgc attgatgact gttggatggc tccccaaaga 300  
gattcagaag gcagacttca ggcagaccct cagcgcttcc ctcattggat tcgccagcta 360  
gctaattatg ttcacagcaa aggactgaag ctagggattt atgcagatgt tggaaataaa 420  
acctgcgcag gcttccctgg gagttttgga tactacgaca ttgatgccca gacctttgct 480  
gactggggag tagatctgct aaaatttgat gggtgttact gtgacagttt ggaaaatttg 540  
gcagatgggt ataagcacat gtccttggcc ctgaatagga ctggcagaag cattgtgtac 600  
tcctgtgagt ggctcttcta tatgtggccc tttcaaaagc ccaattatac agaaatccga 660  
cagtactgca atcactggcg aaattttgct gacattgatg attcctggaa aagtataaag 720  
agtatcttgg actggacatc ttttaaccag gagagaattg ttgatgttgc tggaccaggg 780  
ggttggaatg acccagatat gttagtattt ggcaactttg gcctcagctg gaatcagcaa 840  
gtaactcaga tggccctctg ggctatcatg gctgctcctt tattcatgtc taatgacctc 900  
cgacacatca gccctcaagc caaagctctc cttcaggata aggacgtaat tgccatcaat 960  
caggaccctt tgggcaagca aggttaccag cttagacagg gagacaactt tgaagtgtgg 1020  
gaacgacctc tctcaggctt agcctgggct gtagctatga taaaccggca ggagattggg 1080  
ggacctcgct cttataccat cgcagttgct tcctgggta aaggagtggc ctgtaatcct 1140  
gcctgcttca tcacacagct cctccctgtg aaaaggaagc tagggttcta tgaatggact 1200  
tcaagggtta gaagtcacat aaatcccaca ggcactgttt tgcttcagct agaaaataca 1260  
atgcagatgt cattaaaaga cttactttaa 1290

<210> 68  
<211> 429  
<212> PRT  
<213> Homo sapiens

<400> 68  
 Met Gln Leu Arg Asn Pro Glu Leu His Leu Gly Cys Ala Leu Ala Leu  
 1 5 10 15  
 Arg Phe Leu Ala Leu Val Ser Trp Asp Ile Pro Gly Ala Arg Ala Leu  
 20 25 30  
 Asp Asn Gly Leu Ala Arg Thr Pro Thr Met Gly Trp Leu His Trp Glu  
 35 40 45  
 Arg Phe Met Cys Asn Leu Asp Cys Gln Glu Glu Pro Asp Ser Cys Ile  
 50 55 60  
 Ser Glu Lys Leu Phe Met Glu Met Ala Glu Leu Met Val Ser Glu Gly  
 65 70 75 80  
 Trp Lys Asp Ala Gly Tyr Glu Tyr Leu Cys Ile Asp Asp Cys Trp Met  
 85 90 95  
 Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln Arg  
 100 105 110  
 Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys Gly  
 115 120 125  
 Leu Lys Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala Gly  
 130 135 140  
 Phe Pro Gly Ser Phe Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe Ala  
 145 150 155 160  
 Asp Trp Gly Val Asp Leu Leu Lys Phe Asp Gly Cys Tyr Cys Asp Ser  
 165 170 175  
 Leu Glu Asn Leu Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu Asn  
 180 185 190  
 Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr Met  
 195 200 205  
 Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys Asn  
 210 215 220  
 His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile Lys  
 225 230 235 240  
 Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp Val  
 245 250 255  
 Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly Asn  
 260 265 270  
 Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala Leu Trp Ala  
 275 280 285  
 Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu Arg His Ile Ser  
 290 295 300  
 Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala Ile Asn  
 305 310 315 320

Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp Asn  
 325 330 335

Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val Ala  
 340 345 350

Met Ile Asn Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile Ala  
 355 360 365

Val Ala Ser Leu Gly Lys Gly Val Ala Cys Asn Pro Ala Cys Phe Ile  
 370 375 380

Thr Gln Leu Leu Pro Val Lys Arg Lys Leu Gly Phe Tyr Glu Trp Thr  
 385 390 395 400

Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val Leu Leu Gln  
 405 410 415

Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu  
 420 425

<210> 69  
 <211> 351  
 <212> DNA  
 <213> Homo sapiens

<400> 69  
 atggattact acagaaaata tgcagctatc tttctgggtca cattgtcgggt gtttctgcat 60  
 gttctccatt ccgctcctga tgtgcaggat tgcccagaat gcacgctaca ggaaaaccca 120  
 ttcttctccc agccgggtgc cccaatactt cagtgcattgg gctgctgctt ctctagagca 180  
 tatccactc cactaaggtc caagaagacg atgttggtcc aaaagaacgt cacctcagag 240  
 tccacttgct gtgtagctaa atcatataac agggtcacag taatggggggg tttcaaagtg 300  
 gagaaccaca cggcgtgcc ctgcagtact tgttattatc acaaattctta a 351

<210> 70  
 <211> 116  
 <212> PRT  
 <213> Homo sapiens

<400> 70  
 Met Asp Tyr Tyr Arg Lys Tyr Ala Ala Ile Phe Leu Val Thr Leu Ser  
 1 5 10 15

Val Phe Leu His Val Leu His Ser Ala Pro Asp Val Gln Asp Cys Pro  
 20 25 30

Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro  
 35 40 45

Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro  
 50 55 60

Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu  
 65 70 75 80

Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met Gly  
85 90 95

Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr  
100 105 110

Tyr His Lys Ser  
115

<210> 71  
<211> 498  
<212> DNA  
<213> Homo sapiens

<400> 71  
atggagatgt tccaggggct gctgctgttg ctgctgctga gcatgggagg gacatgggca 60  
tccaaggagc cgcttcggcc acggtgccgc cccatcaatg ccaccctggc tgtggagaag 120  
gagggctgcc ccgtgtgcat caccgtcaac accaccatct gtgccggcta ctgccccacc 180  
atgaccgcgc tgcgtcaggg ggtcctgccg gccctgcctc aggtgggtgtg caactaccgc 240  
gatgtgcgct tcgagtccat ccggctccct ggctgccgc gcggcgtaga ccccggtggtc 300  
tcctacgccg tggtctcag ctgtcaatgt gcaactgtcc gccgcagcac cactgactgc 360  
gggggtccca aggaccacc cttgacctgt gatgaccccc gcttcaggga ctctcttcc 420  
tcaaaggccc ctccccccag ccttccaagc ccaccccgac tcccggggcc ctgggacacc 480  
ccgacccctc cacaataa 498

<210> 72  
<211> 165  
<212> PRT  
<213> Homo sapiens

<400> 72  
Met Glu Met Phe Gln Gly Leu Leu Leu Leu Leu Leu Ser Met Gly  
1 5 10 15  
Gly Thr Trp Ala Ser Lys Glu Pro Leu Arg Pro Arg Cys Arg Pro Ile  
20 25 30  
Asn Ala Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr  
35 40 45  
Val Asn Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val  
50 55 60  
Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg  
65 70 75 80  
Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val  
85 90 95  
Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu  
100 105 110  
Cys Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu

115                      120                      125  
 Thr Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro  
 130                      135                      140  
 Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr  
 145                      150                      155                      160  
 Pro Ile Leu Pro Gln  
 165

<210> 73  
 <211> 165  
 <212> PRT  
 <213> Homo sapiens

<400> 73  
 Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu  
 1                      5                      10                      15  
 Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His  
 20                      25                      30  
 Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
 35                      40                      45  
 Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp  
 50                      55                      60  
 Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu  
 65                      70                      75                      80  
 Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp  
 85                      90                      95  
 Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu  
 100                      105                      110  
 Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala  
 115                      120                      125  
 Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val  
 130                      135                      140  
 Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala  
 145                      150                      155                      160  
 Cys Arg Thr Gly Asp  
 165

<210> 74  
 <211> 588  
 <212> DNA  
 <213> Homo sapiens

<400> 74  
 atggccctcc tgttccctct actggcagcc ctagtgatga ccagctatag ccctgttgga 60  
 tctctgggct gtgatctgcc tcagaacat ggcctactta gcaggaacac cttggtgctt 120  
 ctgcacaaaa tgaggagaat ctcccccttc ttgtgtctca aggacagaag agacttcagg 180

ttccccagg agatggtaaa agggagccag ttgcagaagg cccatgtcat gtctgtcctc 240  
 catgagatgc tgcagcagat cttcagcctc ttccacacag agcgctcctc tgctgcctgg 300  
 aacatgaccc tcctagacca actccacact ggacttcacg agcaactgca acacctggag 360  
 acctgcttgc tgcaggtagt gggagaagga gaatctgctg gggcaattag cagccctgca 420  
 ctgaccttga ggaggtactt ccaggggaatc cgtgtctacc tgaaagagaa gaaatacagc 480  
 gactgtgcct gggaagtgtg cagaatggaa atcatgaaat ccttgttctt atcaacaaac 540  
 atgcaagaaa gactgagaag taaagataga gacctgggct catcttga 588

<210> 75  
 <211> 195  
 <212> PRT  
 <213> Homo sapiens

<400> 75  
 Met Ala Leu Leu Phe Pro Leu Leu Ala Ala Leu Val Met Thr Ser Tyr  
 1 5 10 15  
 Ser Pro Val Gly Ser Leu Gly Cys Asp Leu Pro Gln Asn His Gly Leu  
 20 25 30  
 Leu Ser Arg Asn Thr Leu Val Leu Leu His Gln Met Arg Arg Ile Ser  
 35 40 45  
 Pro Phe Leu Cys Leu Lys Asp Arg Arg Asp Phe Arg Phe Pro Gln Glu  
 50 55 60  
 Met Val Lys Gly Ser Gln Leu Gln Lys Ala His Val Met Ser Val Leu  
 65 70 75 80  
 His Glu Met Leu Gln Gln Ile Phe Ser Leu Phe His Thr Glu Arg Ser  
 85 90 95  
 Ser Ala Ala Trp Asn Met Thr Leu Leu Asp Gln Leu His Thr Gly Leu  
 100 105 110  
 His Gln Gln Leu Gln His Leu Glu Thr Cys Leu Leu Gln Val Val Gly  
 115 120 125  
 Glu Gly Glu Ser Ala Gly Ala Ile Ser Ser Pro Ala Leu Thr Leu Arg  
 130 135 140  
 Arg Tyr Phe Gln Gly Ile Arg Val Tyr Leu Lys Glu Lys Lys Tyr Ser  
 145 150 155 160  
 Asp Cys Ala Trp Glu Val Val Arg Met Glu Ile Met Lys Ser Leu Phe  
 165 170 175  
 Leu Ser Thr Asn Met Gln Glu Arg Leu Arg Ser Lys Asp Arg Asp Leu  
 180 185 190  
 Gly Ser Ser  
 195